Journal of Embryology and Experimental Morphology

VOLUME 4 *June 1956* PART 2

PUBLISHED FOR THE COMPANY OF BIOLOGISTS LIMITED

OXFORD : AT THE CLARENDON PRESS

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GEOFFREY CUMBERLEGE
OXFORD UNIVERSITY PRESS, AMEN HOUSE, LONDON, E.C.4

Growth and Function in the Foetal Liver

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INTRODUCTION

MANY investigators have demonstrated the decline which takes place in the relative weight of the liver during the development of the mammalian foetus; Carlyle (1945) and Wallace (1945) in the sheep, Jackson (1909) in man, Lowrey (1911) in the pig, Williamson (1948) and Addis & Gray (1950) in the rat, Latimer & Corder (1948) in the dog, and Latimer (1948) in the cat. The present work is an attempt to find the cause underlying this decline. Since the foetal liver contains not only hepatic tissue but also much haemopoietic tissue, it was first necessary to determine whether the decrease in the relative liver-weight represents a real diminution of true hepatic tissue or whether it simply reflects the progressive shift of haemopoiesis from liver to skeleton. In order to test these alternatives a method was devised for determining the total amount of true hepatic tissue, i.e. the total number of hepatic cells, in a given foetal liver, and applied to a series of foetuses covering the developmental period.

MATERIAL AND METHODS

The material investigated was a series of fifty-one sheep foetuses ranging in length from 28 to 530 mm. These were collected from the dams immediately after slaughter. Dams were of many different breeds. The following measurements were made.

1. Forehead-rump length. This was measured to the nearest millimetre using the technique of Winters & Feuffel (1936).
2. Body-weight. Surface water was removed and the foetus weighed with the umbilical cord cut short.
3. Weight of liver. The liver was dissected clear of fascia and the gall bladder removed. Weighings were made after removal of surface water with blotting paper.
4. Volume of liver. Before this measurement the liver was fixed in 10 per cent. formalin. In some cases the liver was placed in a syphon apparatus and the volume of the displaced water measured. In others the volume was determined by successive weighings in air and water.

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[J. Embrol. exp. Morph. Vol. 4, Part 2, pp. 97–109, June 1956]

The forehead-rump length was used to calculate the age of each embryo by means of the chart constructed from collected data by Barcroft (1946). This chart combines data obtained from many different breeds. From the scatter of the data given by Barcroft it may be inferred that the probable errors are as follows:

Forehead-rump length	*Estimated age*	*Standard error*	*Coeff. of var.*
mm.	days	days	%
45	42	1·5	3·6
100	55	1·3	2·4
350	114	5·3	4·6
450	138	8·3	6·0

Preparation of liver

A rectangular block was cut from the fixed liver, one of its faces cut with parallel edges, and the length of this face measured with an accuracy of 0·1 mm. by means of a microscope with a movable stage fitted with verniers. The block was dehydrated, embedded in paraffin, and sections cut at 10 μ. The measured edge on the remainder of the block was remeasured by the same method and the linear shrinkage factor,

$$\frac{\text{length after embedding}}{\text{length after fixation}}$$

was calculated. The volume shrinkage factor,

$$\frac{\text{volume after embedding}}{\text{volume after fixation}}$$

was calculated as the cube of the linear shrinkage factor. Six blocks were prepared in a batch and the average of the individual shrinkage factors was applied to the whole batch. The standard deviation of the average was used to estimate the error.

Measurement of section thickness

Sections were stained with haematoxylin and eosin and mounted in DPX. The thickness was then determined by focusing on the upper and lower surfaces of the section using a Leitz Ortholux microscope with an oil-immersion apochromatic objective $\times 90$, N.A. 1·30, and $\times 12$ periplanatic eyepiece. Readings were taken to the nearest 0·5 μ. The depth of focus of the optical combination is given by the maker as 0·8 μ and this figure was subtracted from all the thickness measurements made. Three independent determinations of thickness were made on a small marked part of each section. The lowest and highest of these in general differed by not more than 2 μ. The standard error of the average of the three measurements was within 0·5 μ in all cases. The method of Marengo (1944) was found to be unsuitable for this investigation owing to the considerable variation in the thickness of sections cut at the same time on the same microtome and to

the fact that the thickness measurements would have to be made on sections other than those used for subsequent counting.

Hepatic cell count

The number of hepatic cell nuclei in each of two areas of a section was counted using a × 90 oil-immersion objective. Both of the chosen areas lay within the marked part of the section whose thickness had been determined. The areas were fixed by a micrometer grid in the eyepiece of the microscope which was later calibrated against a stage micrometer. The method normally used in haemocytometer counts was employed and every hepatic nucleus or fragment of nucleus which came within the limits was included. The areas counted were selected at random. If, for instance, the area lay partly over a large blood-vessel the hepatic nuclear count for the area was correspondingly diminished and in extreme cases became zero. All such counts were included as contributing to the final result. From the figures of the hepatic nuclear count, the total area counted in each section, and the measured thickness of the section, the corresponding number of hepatic nuclei per cubic centimetre of liver was calculated for each section. This concentration of hepatic nuclei may be assumed to represent the concentration of hepatic cells, since according to Wilson & Leduc (1948) binuclearity is rare in hepatic cells in the prenatal period. In the calculation of the concentration, the Floderus–Abercrombie correction was employed to take account of the effect of the size of the nucleus on the value of concentration obtained (Floderus, 1944; Abercrombie, 1946). To apply this correction the average width of 20 nuclei was measured, 10 measured in the direction of compression of the section and 10 at right angles to the direction of compression. Nuclei were chosen at random but measurements were made only on those whose greatest diameters lay within the section, as seen by focusing up and down. A value for the concentration of hepatic cells was thus obtained for each of six sections of each liver. The mean of these values was taken as the average concentration of hepatic cells throughout the liver. Since the counts of hepatic cells were made at random irrespective of the presence of other tissues in the field, the value of the average concentration of hepatic cells applies to the volume of the liver as a whole with all its component tissues including blood-vessels.

The scatter of the six sectional concentration values was used as an index, both of the error in the method employed and of the real variation of cell concentration in the tissue. The coefficients of variation of the averages of the six sectional concentration values for each liver ranged from 1·7 to 9·8 per cent. with an average value of 5·1 per cent.

For example, the calculations for foetus No. 37 were performed as in the Table on p. 100.

The value of the average cell concentration was corrected for shrinkage of the block in the processes of preparation by applying the volume shrinkage factor previously determined. The term, 'section compression', as normally used

implies rather a distortion than a true volume compression of the tissue, which seems unlikely in view of the incompressibility of the paraffin embedding medium. Experiment indicated that 'section compression' does not change section volume and thus does not affect the observed cell concentration.

Total area counted per section (two fields) $= 3{\cdot}50 \times 10^4$ sq. μ
Average nuclear diameter $= 5{\cdot}9\,\mu$

Sect. No.	*Total count*	*Thickness*	*Concentration of hepatic cells (after Floderus–Abercrombie correction)*
		μ	
1	100	10·7	$1{\cdot}72 \times 10^8$/c.c.
2	92	10·5	$1{\cdot}60 \times 10^8$/c.c.
3	105	11·0	$1{\cdot}78 \times 10^8$/c.c.
4	93	10·5	$1{\cdot}60 \times 10^8$/c.c.
5	93	10·2	$1{\cdot}65 \times 10^8$/c.c.
6	68	10·5	$1{\cdot}18 \times 10^8$/c.c.

Average concentration of hepatic cells $= 1{\cdot}59 \times 10^8$/c.c.
Standard deviation of average of concentration values $= 0{\cdot}086 \times 10^8$/c.c.
Coefficient of variation $= 5{\cdot}4\%$

(*Note*—In Section 6 the presence of a blood-vessel in the field counted caused a low count and therefore a low figure for concentration.)

The average concentration of hepatic cells in each liver was multiplied by the volume of the liver, calculated from the weight using the modal value of the specific gravity, 1·045. This was found to be practically constant in livers of all stages of development; deviations from the modal value were randomly distributed and were attributed to errors in measurement. The product of concentration and volume thus obtained was taken as an estimate of the total number of hepatic cells in each liver.

The error involved in this estimate was composed of the errors in the average concentration and in the shrinkage factor used to correct it, the error in the determination of weight being negligible in comparison. The coefficients of variation of the shrinkage factors ranged from 2·4 to 5·6 per cent. These, when combined with the coefficients of variation of the cell concentrations already given, resulted in values of the error in the estimate of total hepatic cell population ranging from 3·0 to 10·2 per cent. with an average value of 6·2 per cent.

The standard error of estimate indicated by the scatter diagram of relative hepatic cell population (see below) plotted against foetal age is approximately $1{\cdot}0 \times 10^8$/100 g. of body-weight which gives a coefficient of variation of estimate between 10 and 17 per cent. Since this includes the effects of individual variation along with those of error, the two different methods of computing the error lead to compatible results.

The relative hepatic cell population, that is, the number of hepatic cells present for every 100 g. of body-weight, was then calculated thus:

relative hepatic cell population =

$$\frac{\text{total hepatic cell population}}{\text{body-weight in grammes}} \times 100.$$

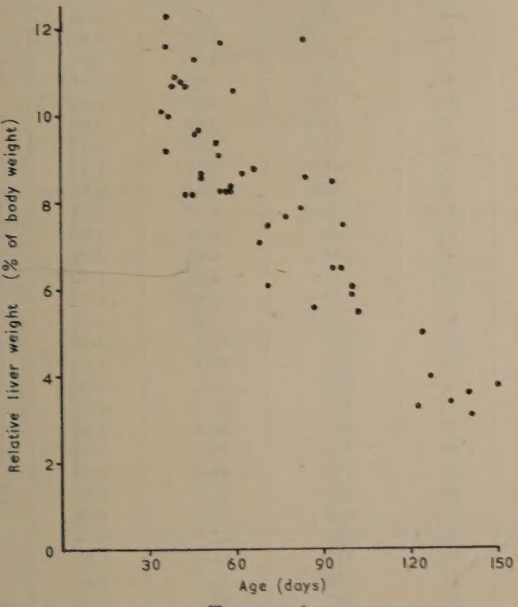
This quantity is comparable with the relative liver-weight as may be seen from the formula

$$\text{relative liver-weight} = \frac{\text{weight of liver in grammes}}{\text{body-weight in grammes}} \times 100.$$

But the relative hepatic cell population expresses the proportion of true hepatic tissue in the body more accurately than the relative liver-weight since the latter takes account of both hepatic and non-hepatic tissue in the liver.

RESULTS

The results are shown in Table 1. Text-fig. 1 shows the relative liver-weight plotted against the age of the foetus. At the 35th day of development the liver forms 11 per cent. of the body-weight, while at term (150 days) it forms only



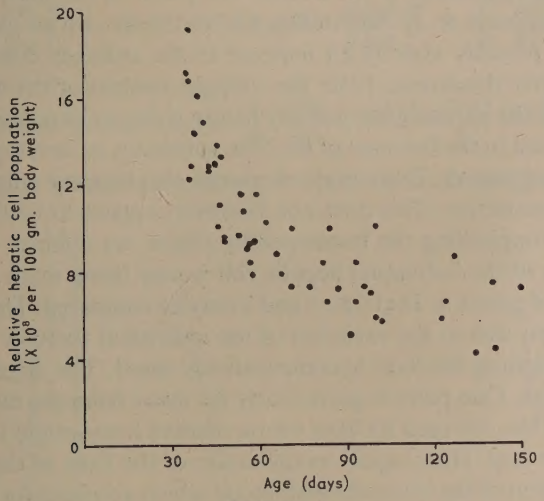
TEXT-FIG. 1

between 3 and 4 per cent. This decline confirms the results of previous workers (Carlyle, 1945; Wallace, 1945). Text-fig. 2 shows the relative hepatic cell population plotted against age. At the 35th day there are on average 16×10^8 hepatic cells present for every 100 g. of body-weight; at term only 6×10^8 / 100 g. of body-weight. It is thus evident that the diminution shown by the relative liver-weight

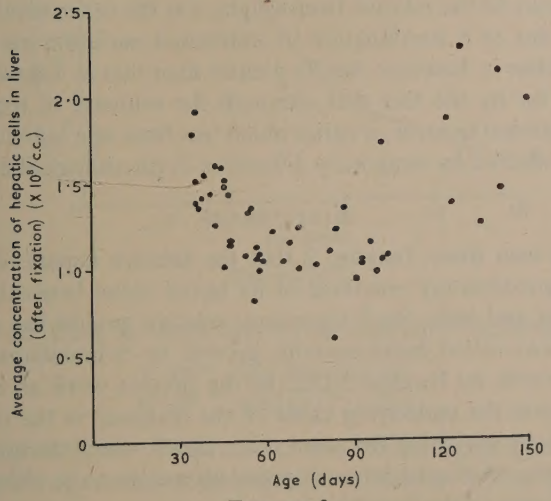
TABLE 1

	Forehead-rump, length	*Estimated age*	*Body-weight*	*Liver-weight*	*Average concentration of hepatic cells in fixed liver*	*Total hepatic cell population*	*Coefficient of variation of population estimate*
	(mm.)	(days)	(g.)	(g.)	($\times 10^8$/c.c.)	($\times 10^8$)	(%)
1	28	35	1·39	0·14	1·78	0·24	4·9
2	30	36	1·95	0·24	1·92	0·38	6·8
3	31	36	2·22	0·20	1·39	0·27	7·2
4	31	36	2·16	0·25	1·52	0·36	6·6
5	32	37	2·56	0·25	1·36	0·33	6·9
6	36	38	3·75	0·40	1·42	0·54	8·3
7	41	39	5·20	0·57	1·55	0·84	7·7
8	44	41	5·48	0·59	1·44	0·82	7·4
9	48	43	5·54	0·59	1·26	0·71	6·2
10	49	43	5·10	0·42	1·61	0·64	3·0
11	57	45	8·80	0·72	1·59	1·10	6·4
12	59	46	10·5	0·75	1·48	1·06	5·5
13	59	46	10·9	1·23	1·03	1·21	5·8
14	60	46	7·74	0·74	1·52	1·08	5·5
15	65	47	15·1	1·46	1·44	2·01	5·6
16	67	48	17·9	1·53	1·17	1·71	7·0
17	70	48	18·5	1·61	1·15	1·77	7·1
18	90	53	31·9	2·99	1·08	3·10	4·0
19	95	54	43·5	3·97	1·33	5·04	9·4
20	98	55	45·0	3·75	1·35	4·84	5·6
21	100	56	50·0	5·82	0·82	4·54	6·0
22	103	57	52·0	4·33	1·13	4·81	4·9
23	106	57	48·9	4·06	1·06	4·11	4·2
24	107	58	58·5	4·88	0·99	4·60	3·4
25	108	58	53·5	4·47	1·18	5·04	4·2
26	114	59	71·5	7·60	1·15	8·33	7·4
27	123	62	81·2	7·10	1·22	8·30	4·5
28	142	66	127	11·2	1·04	11·2	8·0
29	150	68	151	10·7	1·16	11·9	4·9
30	160	71	176	13·2	1·00	12·7	5·2
31	161	71	181	11·1	1·24	13·1	4·9
32	183	77	291	22·5	1·02	21·8	8·8
33	205	82	326	25·7	1·11	27·3	5·1
34	208	83	376	44·2	0·59	25·0	5·4
35	215	84	386	32·9	1·23	38·6	6·3
36	225	87	492	27·6	1·36	35·2	8·3
37	240	91	746	42·4	0·94	37·8	6·8
38	250	93	722	61·4	1·03	60·6	7·5
39	250	93	758	49·6	1·05	50·0	5·6
40	260	96	752	49·2	1·16	54·6	8·5
41	270	98	795	59·5	0·98	55·4	7·2
42	277	100	871	53·0	1·73	87·5	10·2
43	278	100	921	53·9	1·06	54·5	5·4
44	290	102	1,097	60·6	1·08	62·5	3·5
45	365	122	1,555	50·6	1·87	90·4	5·8
46	375	124	1,985	98·7	1·38	130	8·4
47	385	127	1,811	72·6	2·28	158	4·7
48	415	134	2,775	94·8	1·27	115	5·9
49	440	140	2,690	98·0	2·14	216	6·1
50	445	141	3,170	97·8	1·47	138	5·7
51	530	150	6,050	230	1·98	436	8·6

is not due solely to a decrease in the amount of haemopoietic tissue in the liver but that in the course of development there is a real diminution in the proportion



TEXT-FIG. 2



TEXT-FIG. 3

of true hepatic tissue present in the foetus. In fact the parallel diminution of cell number and liver-weight is due to the comparative constancy of the concentration of hepatic cells in the liver. Text-fig. 3 shows the average concentration of hepatic cells in the fixed liver (the shrinkage factor having been applied) plotted against the age. There is no statistically significant difference ($0{\cdot}25 > P > 0{\cdot}1$)

between the concentrations at the beginning and end of the period of development investigated although there is a fall in concentration in the intervening period which is significant ($P < 0{\cdot}001$). The place of the diminishing haemopoietic tissue appears to be fully taken up, and more, by an expansion of the sinusoids and possibly also by an increase in the collagen content. As in the regenerating liver (Harkness, 1952) the collagen content of the developing liver may lag behind the parenchyme initially before rising to its definitive value. The increase observed in the firmness of the liver substance as development proceeds supports this suggestion. There may, of course, also be some enlargement of the hepatic cells themselves. This does not, however, appear to be the most important factor in supplanting the haemopoietic tissue. An attempt to measure the changes in size of the individual hepatic cell is now being made.

The scatter of points in Text-figs. 1 and 2 may be compared. That in Text-fig. 1 is almost entirely due to the variation of the individual foetuses since the error involved in weighing the liver is comparatively small. The degree of scatter is nevertheless high. One point is particularly far away from the rest, representing a foetus (serial No. 34) aged 83 days whose relative liver-weight is exceptionally high—11·8 per cent. Histological examination of the liver of this foetus shows intense congestion of the sinusoids with blood which accounts for the high value. In general, similar temporary irregularities may produce the considerable variation which occurs in the relative liver-weight. On the other hand, the scatter in Text-fig. 2 is due to a combination of individual variation and experimental errors. The scatter is, however, hardly greater than that in Text-fig. 1. This may be accounted for by the fact that although the estimate of total hepatic cell population is subject to error, it varies much less from one individual to another since it is not affected by temporary difference in physiological conditions.

DISCUSSION

It has been seen from Text-fig. 2 that the relative hepatic cell population declines to approximately one-third of its initial value between the 35th day of development and term. Such inconstant relative growth has been variously explained; it was called heteronomous growth by Schmalhausen (1927) and heterogonic growth by Huxley (1932). In the present work an endeavour was made to elucidate the underlying cause of the decrease in the relative hepatic cell population by searching for some other factor which declines in a similar manner. Thus the relative hepatic cell population came to be compared with the specific growth-rate of the foetal body.

The specific growth-rate was obtained from data of body-weight (W) and age (T) by a simple graphical method. Logarithms were taken of both W and T so as to rectify the resulting curve so far as possible. A 'best line' was drawn through the data and the gradient of this measured directly at short intervals along the log T axis. Each value of the gradient thus measured was then plotted against the mid-value of the log T interval from which it was derived. This resulted in a

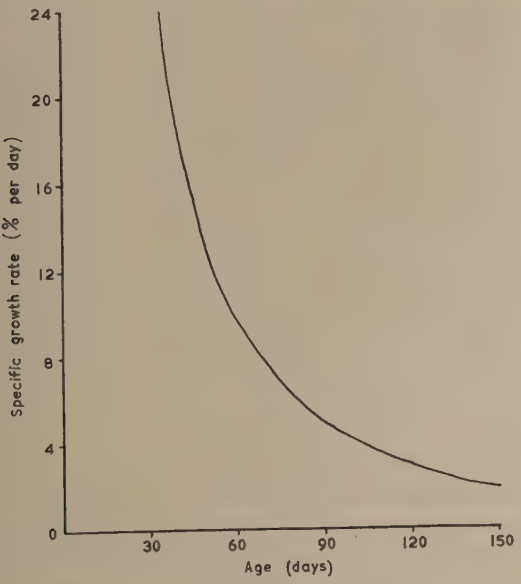
practically linear relationship between the gradient $\delta \log W/\delta \log T$ and $\log T$, whence the following equation was derived:

$$\frac{\delta \log W}{\delta \log T} = 20{\cdot}12 - 7{\cdot}95 \log T \simeq \frac{d \log W}{d \log T} = \frac{T}{W} \cdot \frac{dW}{dT}.$$

Thus

$$\text{specific growth-rate} = \frac{1}{W} \cdot \frac{dW}{dT} = \frac{20{\cdot}12 - 7{\cdot}95 \log T}{T}.$$

The values for the specific growth-rate so obtained are in good agreement with



TEXT-FIG. 4

those which result from the same data by the formula of Schmalhausen (1927):

$$\frac{1}{W} \frac{dW}{dT} = \frac{\log W_2 - \log W_1}{0{\cdot}4343\,(T_2 - T_1)}.$$

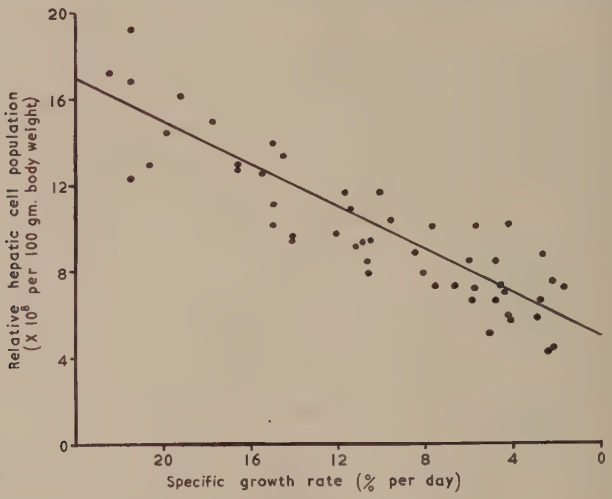
The specific growth-rate of each foetus was thus calculated and the resulting relationship to the age is shown graphically in Text-fig. 4. The specific growth-rate falls from 22·4 per cent. per day at the 35th day to 1·7 per cent. per day at term.

It may be observed that the real specific growth-rate of an individual foetus can only properly be obtained from successive observations of its weight at intervals during its development. Estimates derived by any method from the weights of a series of foetuses are at best only approximations. The fact that the ages were estimated indirectly from the forehead-rump length may be expected

to introduce an error into the estimate of the specific growth-rate but this hardly affects the accuracy of the final result since, as pointed out, error is inevitably already present.

Correlation between relative hepatic cell population and specific growth-rate

The form of the curve of the specific growth-rate shown in Text-fig. 4 is seen to be very similar to that of the relative hepatic cell population in Text-fig. 2.



TEXT-FIG. 5

This suggests a relationship between these two quantities. The presence of such a relationship may be further investigated by plotting the two quantities against one another. The result of this is shown in Text-fig. 5. This reveals a linear correlation between the relative hepatic cell population and the specific growth-rate between the 35th day of development and term. The correlation is expressed fairly accurately by the equation,

$$N = 0{\cdot}5 \times 10^8 R + 5 \times 10^8,$$

where N is the relative hepatic cell population per 100 g. of body-weight and R is the specific growth-rate in per cent. per day. A straight line expressing this equation has been drawn through the data of Text-fig. 5.

This proportionality between the relative hepatic cell population and the specific growth-rate suggests that a definite number of hepatic cells is required to maintain a given rate of growth, i.e. that each 100 g. of foetal tissue requires the activity of $0{\cdot}5 \times 10^8$ hepatic cells for every unit of 1 per cent. per day in its rate of growth. The equation constant, $5 \times 10^8/100$ g. of body-weight, may be taken to be the number of hepatic cells which is required to maintain the normal metabolic processes of a given 100 g. of foetal tissue apart from its growth.

It must, of course, be emphasized that many natural rates decline with increasing age and that the correlation of the relative hepatic cell population with the specific growth-rate does not prove a direct functional connexion between them. It is no more than consistent with the presumption that such a connexion exists.

The assumption of an anabolic function of the liver in growth is not confirmed by any conclusive evidence so far available. Biochemical knowledge at present relates more to dissimilative than to synthetic processes. It is, however, known that synthesis of plasma proteins, fibrinogen, and prothrombin takes place in the liver and it seems not unreasonable to assign to it an important place in the synthetic mechanisms of growth. It may, of course, be assumed that before the appearance of the liver, the comparatively undifferentiated somatic cells are capable of conducting the growth syntheses by themselves. The variation of the number of hepatic cells in response to functional demand may be likened to the hypertrophy of muscle in response to work, or to wasting following disuse.

An excellent illustration of the effect of functional demand in controlling organ size is provided by the work of Walter & Addis (1939). In experiments in which the metabolic- and growth-rates of young rats were raised or lowered by thyroid administration or thyroidectomy, they found that thyroid administration produced a rise and thyroidectomy a fall in the relative weights of the heart, kidney, and liver. The failure of Evans, Simpson, & Li (1948) to find a similar effect after injection of pituitary growth hormone into rats may be due to the fact that the increase they produced in the specific growth-rate was only 0·12 per cent. per day.

Comparison of the total amount of hepatic substance with the functional demands made upon it may be criticized on the ground that the adult liver has a large functional reserve, e.g. that 80 per cent. of it may be removed without any obvious impairment of hepatic function (Bollmann & Mann, 1936). However, there is no reason to suppose that the foetal liver has a similar reserve; indeed, the investigation of Findlay, Higgins, & Stanier (1947) into the cause of icterus neonatorum suggests that the foetal liver has little or no reserve and that the extra demands made on it immediately after birth result in temporary hepatic insufficiency. In any case, as pointed out by Abercrombie (1955), since even the adult liver regenerates rapidly to its normal size after partial hepatectomy, there must be at least one function for which the liver has no reserve. From experiments in parabiosis and tissue culture it seems likely that this function is the utilization or production of some substance whose blood-level controls the size of the liver (Bucher, Scott, & Aub, 1951; Glinos & Gey, 1952). The regulation of the amount of hepatic tissue in the foetus may well be attributed to a similar humoral mechanism.

One fact inconsistent with the above theory may be noted. According to the work of Schmalhausen (1926) on the chick and of Kaufman (1930) on the chick and pigeon, the relative liver-weight increases during embryonic life while the specific growth-rate as usual declines. This effect may of course be due to the

growth of non-hepatic tissue in the liver, and it will be necessary to discover the behaviour of the true hepatic tissue in the chick liver. In the dog-fish the relative liver-weight declines in the prenatal period as in mammals (Kearney, 1914).

Latimer (1948) has shown that in the foetal cat nine organs reach their maximum relative weight at the close of the embryonic stage and thereafter undergo a relative decline; these are the heart, liver, pituitary, thyroid, brain, spinal cord, eyeballs, suprarenals, and ovaries. Of these organs the brain, spinal cord, and eyeballs have no obvious connexion with bodily growth and their decline must be put down to other factors. The ovaries are subject to control by the pituitary gonadotrophic hormones. The relative decline of the heart, pituitary, thyroid, and suprarenals as well as of the liver may, however, be related to the concurrent decrease in the specific growth-rate. This question requires further investigation.

There is one important consequence of this theory of liver growth which provides an opportunity of testing it. The approach which has been used to the problem of heterogonic liver growth is a physiological one, since it has been assumed that the amount of hepatic functional capacity present in the foetus at any time is determined by the functional demands made upon it. Thus the decline in the relative hepatic cell population may be due either (1) to an increase in the functional capacity of the individual hepatic cell, functional demand remaining constant, or (2) to a fall in functional demand. If the decline is to be accounted for mainly by a fall in functional demand due to decrease in the specific growth-rate, it must follow that the individual hepatic cell of the sheep foetus does not change its metabolic activity significantly between the 35th day of development and term, i.e. that it is functionally almost fully mature before the 35th day. An attempt is now being made to test this conclusion.

SUMMARY

1. A method is described for estimating the total population of hepatic cells in the liver of a sheep foetus.

2. Use of this method has shown that during development there is a real decrease in the relative number of hepatic cells in the sheep foetus.

3. The relative number of hepatic cells in the foetus is directly proportional to the specific growth-rate of the foetal body.

4. It is suggested that the relative number of hepatic cells in the foetus is determined by functional requirements, partly to perform normal metabolic processes and partly to maintain growth.

ACKNOWLEDGEMENTS

I am indebted to Professor G. M. Wyburn for his encouragement and advice in the course of this work. For much helpful advice on the mathematical and statistical treatment of the data, I must thank Dr. S. D. Silvey of the Mathematics Department, University of Glasgow.

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(Manuscript received 30:vi:55)

Teratogenic Effects of Trypan Blue on Amphibian Embryos

by C. H. WADDINGTON *and* M. M. PERRY[1]

From the Institute of Animal Genetics, Edinburgh

WITH ONE PLATE

Dedicated to
PROFESSOR ALFRED KÜHN
on his 70th birthday, 22nd April 1955

INTRODUCTION

CONSIDERABLE interest has been aroused by the report of Gillman, Gilbert, Gillman, & Spence (1948) that the vital dye trypan blue when injected into the pregnant rat brings about the appearance of various abnormalities in the offspring. The mammal embryo is notoriously difficult to attack experimentally, and trypan blue therefore seems to provide a valuable tool which, moreover, might throw light on the mechanisms of the important effects which are known in some cases to be produced on the foetus by pathological states in the mother. Further studies on the effects of injected trypan blue on mammalian embryos have therefore been made by Hamburgh (1952, 1954), Waddington & Carter (1952, 1953), and Murakami and his collaborators (1952, 1954), all of whom worked on the mouse, and by Harm (1954) who studied the rabbit. All these authors agree, on the whole, in the type of results which they have obtained, although there are some minor differences between their reports which will be considered later. All the investigations, however, have revealed a rather diversified spectrum of malformations from which it is by no means easy to deduce whether trypan blue has one primary effect (and if so, what is its nature) or whether it operates in a number of different and rather unspecific ways. Moreover, the method of administration employed in mammals, namely injection into the maternal blood-stream, leaves it open whether trypan blue can have a direct effect on the embryo or whether its action is always meditated through some alteration produced in substances supplied by the mother to the foetus.

In the hope of obtaining further information on these points trypan blue has been administered to amphibian embryos of a number of types.

The dye (taken from the same purified samples, supplied by Messrs. Imperial

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[J. Embryol exp. Morph. Vol. 4, Part 2, pp. 110–19, June 1956]

Chemical Industries, Dye Stuff Division Ltd., which were used in the investigation described by Waddington & Carter, 1953) was made into a 1 per cent. solution in distilled water and this solution further diluted with one-fifth Holtfreter solution. Embryos of *Xenopus*, Axolotl, and *Triturus palmatus* and *alpestris* were placed in solutions of appropriate dilution for various lengths of time before being transferred into one-tenth Holtfreter. The embryos were usually left in their vitelline membranes. A total of about 700 embryos were studied, about 80 being sectioned for microscopical examination.

DESCRIPTION OF RESULTS

1. *Xenopus*

Eggs and embryos of *Xenopus*, removed from the jelly but still within the vitelline membrane, were treated with solutions of the dye in one-fifth normal Holtfreter solution. After some preliminary experiments a dye concentration of 0·025 per cent. was found to be suitable. Eggs of various ages from the early cleavage to the neurula were left in this for periods of 24 or 48 hours, after which they were transferred to one-fifth normal Holtfreter solution for further cultivation. Embryos which remained healthy showed only slight signs of staining by the dye, but dead cells coloured intensively. In most batches of eggs subjected to the dye there was considerable variation in the degree of abnormality produced, probably as a result of differences in the degree to which the dye-stuff was taken up by the living material. On the whole the effects were stronger the earlier the stage at which the egg was placed in the dye, and the longer it remained there.

In some of the eggs treated in early stages the process of gastrulation was completely inhibited. The end result of development was the formation of a ball of rather featureless cells, which were heavily yolk-laden and more endoderm-like towards the centre of the mass, and more epidermis-like towards the outer surface, which was often strongly wrinkled. In some regions the superficial layer of tissue was lifted away from the more deeply lying cells leaving a fluid-filled vesicle. In less extreme cases invagination took place at least to some extent. The resulting embryos often exhibited various degrees of reduction of the head, as well as irregularities in the tail, and were very often affected by a severe oedema. An example is shown in Fig. A of the Plate. On sectioning, such embryos showed a variety of abnormalities. Perhaps the most interesting of these consisted in the reduction of the notochord. In some instances (Plate, fig. B) this might be completely absent, the somite mesoderm from each side uniting in the midline to give a very irregularly segmented mass. In such cases the neural tube was also very much reduced, and in the example figured, which is from an embryo which showed very little axial elongation, the epidermis is thickened and wrinkled. In another example (Plate, fig. C), a notochord is present though it is much smaller than normal, and the somite mesoderm unites beneath it. The embryo is oedematous and the tissue of the neural tube is undergoing a peculiar type of degeneration in which many small vacuoles appear among the cells. Similar vacuoles

appear in the mesoderm where, however, they are a normal feature of the histogenesis of the tissue.

The suppression of the notochord in embryos treated before gastrulation is probably brought about by an effect on the processes of regionalization by which the invaginated sheet of mesoderm becomes divided up into a strand of notochord in the middle with somite mesoderm on each side. This process is a 'field phenomenon', as Yamada (1940) in particular has shown, and the effect of trypan blue treatment of early embryos seems to be to reduce the 'potential' of the central strand of mesoderm so that it develops into somite instead of chorda. Even when embryos are not placed in the trypan blue until the end of gastrulation, a certain degree of mesodermalization is sometimes produced; an example of this is shown in Fig. D of the Plate. With still later treatments, the notochord differentiates normally.

The development of the neural system is influenced in several different ways. In the first place, when there is extensive suppression of the notochord the overlying neural system is also extremely reduced. This reduction can probably be regarded as a result of a weakening of the inductive powers of the mesoderm. A different type of action is probably responsible for the production of microcephaly, which seems in most cases to result from an inhibition of the forward movement of the invaginating mesoderm, accompanied by a depression of the anterior part of the inductive field. This is a very usual result of inhibitory agents acting during the process of gastrulation. Trypan blue, however, also exerts a third type of influence on the neural system, namely a toxic effect on the differentiating cells, which in some embryos are obviously unhealthy and show a large number of small non-staining vacuoles in the cytoplasm (Plate, fig. C).

2. *Axolotl*

A small series of axolotl eggs were exposed to 0·025 per cent. trypan blue for $18\frac{1}{2}$ hours in blastula and early gastrula stages. One day after their removal from the dye solution some of the embryos were found to be completely exogastrulated. Even those which developed best exhibited extreme grades of microcephaly, no eyes at all being present (Plate, fig. E). At the posterior end several of the embryos had a large swelling, which sections showed to be composed mainly of notochord. These two effects are probably due to a partial suppression by the trypan blue of the gastrulation movements and of the elongation of the tail-bud. The elongation, particularly of the posterior end, was also inhibited in embryos treated from the late gastrula stage onwards. Sections from other individuals treated as blastulae or young gastrulae showed some clear examples of mesodermalization of the chorda. In extreme cases the chorda was completely absent (Plate, fig. F); in others, although the chorda was present, it was improperly differentiated, the cells remaining full of yolk granules to a much later stage than usual. There were also some morphological abnormalities in the anterior part of the nervous system (Plate, fig. H).

3. Triturus palmatus *and* alpestris

In a series of eggs of two species of *Triturus*, treated with similar concentrations and exposures, the effects were in general less striking, and more suggestive of a general toxicity of the substance than of any specific effects. In some eggs treated from early stages, gastrulation was completely suppressed, the embryos remaining more or less featureless balls of cells. Sometimes these showed a certain thickening of the outer layer along a line which presumably corresponds to the axis. There was little sign of any differentiation of specific types of tissue within this linear thickening. One can perhaps interpret these as embryos in which gastrulation had taken place but in which there was an extreme degree of mesodermalization, leading to the complete suppression of notochord formation and of the induction of the neural system. The embryos which were less severely affected were usually relatively normal, although considerably slowed up in their rate of differentiation. In a few cases there was a slight reduction in the size of the chorda, accompanied by a tendency for the somite mesoderm to unite beneath it; this represents a mild degree of mesodermalization. The embryos are frequently oedematous, but showed little tendency towards microcephaly. The epidermis is often highly abnormal, the cells becoming very large and vacuolated (Plate, fig. G). In the axial region the epidermis, mesoderm, and neural tissue are often not properly separated from one another and in such cases the neural tissue is usually badly differentiated. There were also some examples of disturbed morphogenesis of the neural system, particularly of the anterior region.

DISCUSSION

These experiments show that trypan blue can cause abnormalities in development when administered directly to embryos, and does not need to operate through some intermediary system such as the maternal blood-stream. It remains an open question whether when it is injected into pregnant mammals it is absorbed by the foetus and acts directly on it, or whether it operates by modifying the maternal fluids, but there is certainly no *a priori* reason for making the latter assumption. In mammalian embryos whose mothers have been injected, there is no visible sign of coloration by the dye except in the yolk sac and placenta, and this might suggest that the dye is not affecting the embryo directly. However, there is very little sign of any blue tint in amphibian cells exposed to trypan blue, at least until they become necrotic, when the dye is taken up quite rapidly; it seems, therefore, that the dye can produce a teratogenic effect when its intra-cellular concentration is extremely low.

In the experiments with amphibia a number of different kinds of malformations have been produced. The main types are:

1. *Oedema*

The treated embryos, particularly of *Xenopus*, are frequently extremely swollen and full of fluid in the tissue spaces.

2. *Microcephaly*

The main cause of this would seem to be an inhibition of the gastrulation movements. In embryos treated at the neural plate stage, however, in which the invagination would be complete, there are also signs of bad differentiation of the head, and it seems probable that the anterior part of the neural system is more sensitive than the posterior to some relatively unspecific inhibitory influence on tissue differentiation.

3. *Formation of large masses of notochord at the posterior end*

This effect has been seen both in *Xenopus* and Axolotl. It is presumably due to an inhibition of the elongation of the tail-bud.

4. *Abnormalities of the epidermis*

The cells of this tissue frequently become vacuolated and swollen. The epidermis may also become fused with the underlying mesoderm. It is sometimes thrown into numerous folds and wrinkles, probably as a result of continued growth in an embryo which is itself failing to become elongated.

5. *Vacuolar degeneration of neural tissue and mesoderm*

Various forms of tissue necrosis have been seen, of which this is the most striking. It is particularly marked in *Xenopus*, whereas in *Triturus* a more usual appearance is pycnosis of the nuclei.

6. *Suppression of gastrulation*

In some eggs treated before gastrulation and very severely affected, the entire process of invagination is suppressed and the embryo becomes a more or less featureless lump in which no tissue differentiation takes place. This can be regarded as an extreme form of the disturbances of gastrulation and morphogenetic movement which bring about the microcephaly and posterior enlargement of the chorda described in paragraphs 2 and 3 above.

7. *Mesodermalization of the notochord*

Both in *Xenopus* and Axolotl, but not in the comparatively small series of *Triturus*, examples have been seen of an effect extremely similar to the well-known action of lithium on the amphibian embryo. The notochord is partially or completely suppressed, the material from which it should develop being converted into more lateral mesoderm, of the nature of somite or even pronephros. In such embryos the two rows of somites are usually united across the midline, and the neural tube is also very much reduced, probably as a consequence of the reduction in the axial mesoderm. It is noteworthy that in *Xenopus* even with treatment as late as the end of the gastrula stage there may be some apparent mesodermalization of the notochord, but perhaps this should be compared rather with the 'Chorda blocking' described by Hadorn (1951) than with the normal lithium effects.

The mesodermalizing action of lithium is usually regarded as a relatively specific effect rather than a sign of a general inhibitory action. It has been compared with the vegetalization produced by the same substance on the echinoderm egg. The mechanism of these lithium effects has been considerably discussed (e.g. Lehmann, 1945; Pasteels, 1945; Gustafson, 1950, &c.). One of the most plausible hypotheses has been advanced by Ranzi (cf. 1951; Ranzi & Citterio, 1954). He has described a number of substances which act similarly in vegetalizing the sea-urchin embryo, while there is another group which shows the opposite action, increasing the tendencies towards animal development. It is known that at least one member of the former group, namely lithium itself, produces a mesodermalization of the chorda in amphibia, while some of the latter group (e.g. urea) tend to cause an increase in the size of the amphibian chorda.

Ranzi suggests that both groups of substances act by virtue of their influence on the state of aggregation of protein particles in solution. He finds that vegetalizing substances, at the concentrations at which they are embryologically active, bring about an increase in the viscosity of fibrillar protein solutions, and at the same time render the particles more resistant to the demolizing action of urea. The animalizing substances have the opposite effects. This hypothesis is based primarily on the properties of substances known to act in a vegetalizing manner on echinoderm eggs; rather few such substances have yet been shown to produce mesodermalization in the amphibia. It therefore seemed interesting to test the effect of trypan blue on protein solutions in the manner that Ranzi has previously used. Fortunately, Professor Ranzi himself expressed his willingness to carry out these tests, and we are extremely grateful to him for allowing us to quote the following results.

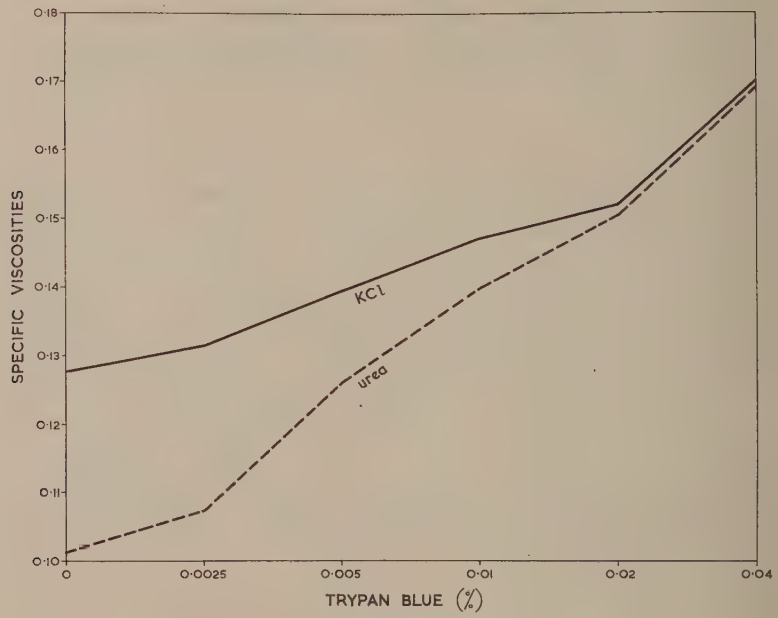
TABLE 1

Specific viscosities of euglobulin. Determined by Prof. S. Ranzi, Milan, by the methods described in Ranzi & Catterio (1954)

Concentration trypan blue percent.	*Viscosities*	
	Protein solution	*Protein solution plus urea*
0·0	0·1276	0·1014
0·0025	0·1314	0·1074
0·005	0·1393	0·1258
0·01	0·1469	0·1396
0·02	0·1518	0·1503
0·04	0·1700	0·1690

Euglobulin $a + b$ from frogs' eggs was prepared according to the method of Ranzi & Citterio (1954). Six samples each of 10 ml. were taken, and to each was

added 10 ml. of 1 M KCl containing various concentrations of trypan blue, so that the concentration of the dye in the final solutions was brought to the values of 0·04 per cent., 0·02 per cent., 0·01 per cent., 0·005 per cent., 0·0025 per cent., 0 per cent. After one night in the cold room, each sample was divided in half. To the first of these 5-ml. samples, 0·6 ml. of 1 M KCl was added, and to the second 0·6 ml. of 30 per cent. urea. Following the methods of Ranzi and Citterio in the paper cited, the viscosities were read 4 hours later. The values obtained are



TEXT-FIG. 1. Specific viscosities of solutions of euglobin *a* and *b* from frogs' eggs, diluted either with KCl or urea, and containing different percentages of trypan blue. Determinations by Prof. S. Ranzi.

shown in Table 1. The effect of trypan blue in increasing the viscosity, and in rendering the protein particles more resistant to the action of urea, is very clearly seen. In fact, if the data are plotted (Text-fig. 1), they exhibit a picture very similar to that found for lithium and shown in fig. 9 of Ranzi & Citterio's paper. There is, then, clear evidence that trypan blue falls into line with what would be expected from it on Ranzi's hypothesis. This can probably be taken as rather strong support for the hypothesis, since trypan blue is a substance of quite different chemical nature from the other vegetalizing agents known.

Although this investigation by Professor Ranzi gives us some basis for understanding the action of trypan blue in causing mesodermalization in the amphibian egg, it must be remembered that the substance has several other effects

which have been enumerated above. It is not possible at present to see how these can all be brought under the same heading as the mesodermalization. It is possible, indeed, that the morphogenetic effects on gastrulation and the elongation of the tail-bud may also be the results of an influence on the physical state of the protein particles in the cytoplasm, but this at present remains mere speculation. The disturbance of the fluid balance leading to oedema, the effects on the epidermis, and the vacuolar degeneration of neural and mesodermal tissues, are probably to be attributed to more general toxic properties of trypan blue, not necessarily immediately related to its influence on protein viscosity.

It will be noted that there is some difference in the incidence of the various types of abnormalities in the different species of amphibia used. Thus mesodermalization was common in *Xenopus* and in the small series of Axolotl but was not seen in *Triturus* species, which showed only rather unspecific effects suggestive of a general toxicity. A comparison of the results of treating amphibian and mammalian embryos also provides evidence that the genetic nature of the material strongly affects the kind of abnormalities produced. In the latter group, few signs have been noticed of a mesodermalization of the notochord, although Murakami, Kameyama, & Kato (1954) have described some embryos which might perhaps be interpreted in this way. Hamburgh (1954) mentions what he calls 'wavy notochords', but this abnormality seems to be due to a general disturbance of growth of the somites rather than to a mesodermalization of the chorda. The production of oedema accompanied by blistering is, however, an effect which is as marked among the mammals as in the amphibia. Waddington & Carter (1953), indeed, suggested that most of the abnormalities in mouse embryos of their strain could be interpreted as secondary results of such disturbances of the fluid balance. In particular, the abnormalities in the tail region seemed often to result from the formation of local accumulations of blood or body fluids. In so far as this is the case, they would not be truly comparable to the abnormalities of the tail-bud region described in the Amphibia, since these appear to be due to a direct effect on the morphogenetic movements. It may be, however, that similar direct effects played a greater part in causing the tail abnormalities of mouse embryos than was previously thought.

There are considerable differences in the effects of trypan blue on the nervous systems of the mammals and the amphibia. In the latter, we have seen striking examples of the production of microcephaly, and in general the effect of the dye is to bring about a reduction of the nervous system. In mammals all workers agree that there are considerable abnormalities in the closure of the neural folds in the period shortly after the administration of the dye. In Waddington & Carter's material (CBA inbred mice) it appeared that embryos affected in this way usually died at the age of about 12 to 13 days. In other stocks many of them survive to later stages. Both Hamburgh and the Japanese workers point out that in such surviving embryos there may be a considerable hypertrophy of the nervous system, involving overgrowth rather than reduction. In some cases, the final

result at birth was a typical pseudencephaly, with an enlarged and unenclosed brain sitting on top of the head like a cap. The frequency of this abnormality seems to vary in different mouse stocks. It amounted to something under 18 per cent. in the Bagg albino strain used by Hamburgh, to about 7 per cent. in an albino strain used by Murakami, to less than 1 per cent. in certain pigmented stocks used by the same author, while it was not seen at all in the CBA's used by Waddington & Carter. It also appeared in the rats studied by Gillman *et al.* but is not described in the fairly small series of abnormalities produced by Harm (1954) in the rabbit.

SUMMARY

1. Trypan blue, at concentrations around 0·025 per cent., was allowed to act on cleavage, gastrula, and early neurula stages of *Xenopus*, Axolotl, and *Triturus alpestris* and *palmatus* for various periods (about 24 or 48 hours).

2. A variety of abnormalities were produced, of which the most striking were: (*a*) Oedema. (*b*) Microcephaly. (*c*) Prevention of elongation of the tail bud. (*d*) Vacuolation and swelling of the epidermal cells and fusion of the epidermis to the mesoderm. (*e*) Degeneration of the neural tissue (and to a lesser extent of the mesoderm) with appearance of vacuoles in the cells. (*f*) Suppression of gastrulation. (*g*) Mesodermalization of the notochord, similar to that produced by lithium.

3. It seems unlikely that all these effects are consequences of one single original action. Some of the abnormalities (particularly (*d*) and (*e*), and to a lesser extent (*b*) and (*g*)) can be produced when the exposure to the dye occurs after gastrulation.

4. In view of the lithium-like effect, Professor S. Ranzi of Milan has tested the action of trypan blue on the viscosity of protein solutions and their resistance to the action of urea, and found that it behaves similarly to other 'vegetalizing' agents.

5. The incidence of these types of abnormality differs in the different amphibia investigated, as it also appears to do in the various stocks of mice which have been studied.

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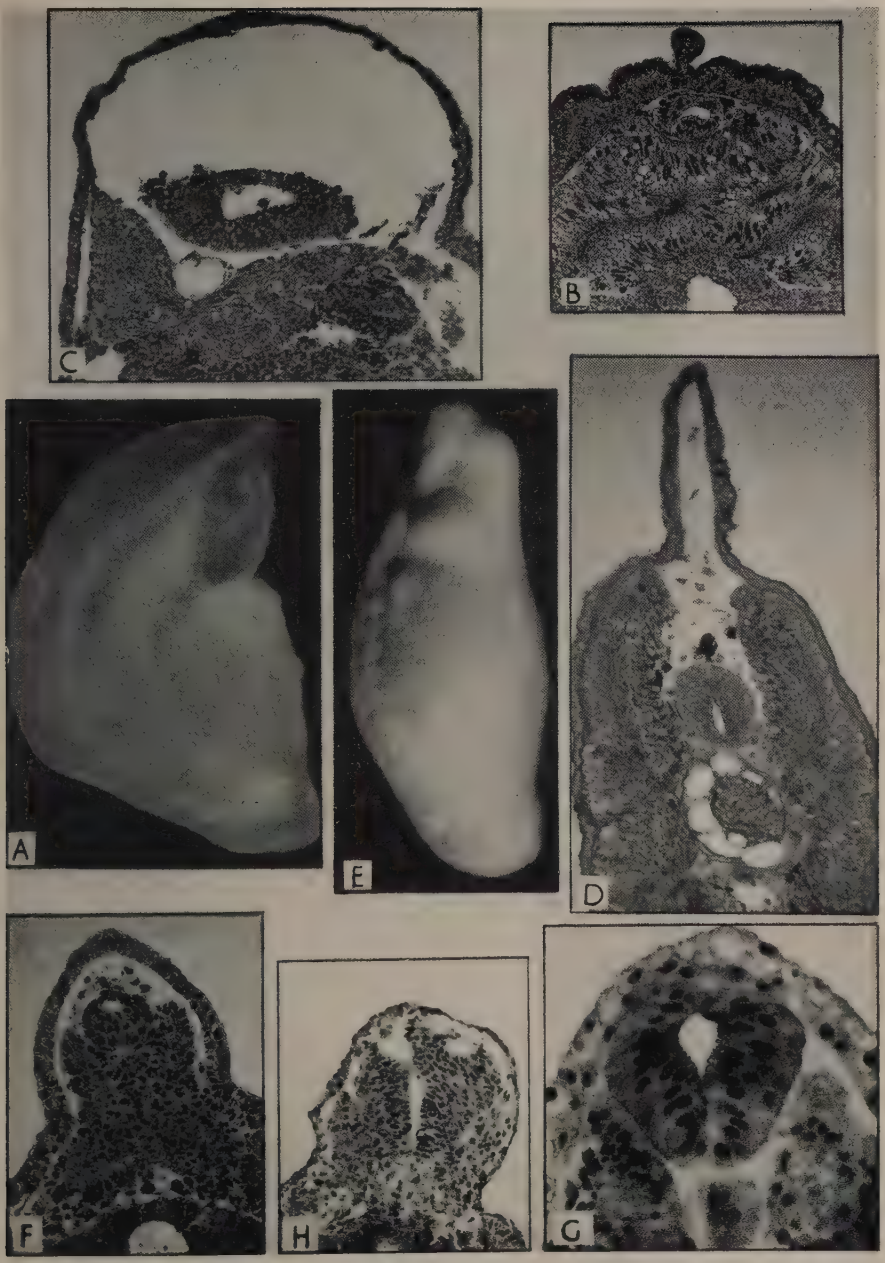

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EXPLANATION OF PLATE

FIG. A. *Xenopus.* 24 hours in 0·025 per cent. trypan blue, from blastula stage; then 4 days in one-fifth Holtfreter.

FIG. B. *Xenopus.* 24 hours in 0·025 per cent. trypan blue from 4-cell stage, then 2 days in one-fifth Holtfreter.

FIG. C. *Xenopus.* 48 hours in 0·025 per cent. trypan blue from late blastula, then 3 days in one-fifth Holtfreter.

FIG. D. *Xenopus.* 24 hours in 0·025 per cent. trypan blue from late gastrula, then 2 days in one-fifth Holtfreter.

FIG. E. Axolotl. 18½ hours in 0·025 per cent. trypan blue from young gastrula, then 4 days in one-half Holtfreter.

FIG. F. Axolotl. 16½ hours in 0·025 per cent. trypan blue from blastula, then 5 days in one-half Holtfreter.

FIG. G. *T. alpestris.* 48 hours in 0·01 per cent. trypan blue from early gastrula.

FIG. H. Axolotl. 16½ hours in 0·025 per cent. trypan blue from blastula, then 5 days in one-half Holtfreter.

(Manuscript received 14:vii:55)

Analysis of the Determination of the Olfactory Placode in *Amblystoma punctatum*

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WITH ONE PLATE

INTRODUCTION

THE analysis of the factors involved in the determination of the olfactory organ or 'nose' in amphibians is still in a preliminary stage. Obviously, like all other ectodermal structures, this organ owes its emergence to inductive stimuli derived from adjacent tissues. In the numerous isolation experiments performed on the gastrula ectoderm of different species olfactory organs never occurred. The earliest stage at which the prospective nose ectoderm was found to be capable of self-determination was the early neurula (Carpenter, 1937; Zwilling, 1940). The question arose as to which of the tissues in the immediate environment of the nose represents its inductor. The tip of the forebrain primordium and/or the thin layer of rostral head mesoderm seemed to be worth serious consideration.

Evidence in favour of the view that the head mesoderm is directly engaged in nose induction is rather slim and inconclusive. This applies especially to the data of Spemann (1912) and Raunich (1950 *a*, *b*) who found that nasal organs differentiate *in situ* after the removal of the anterior part of the neural plate. Transplantation experiments of Carpenter (1937) and Zwilling (1940) showed the nose primordium to be determined shortly before the formation of the neural folds, which indicated to these workers that the head mesoderm is engaged in nose induction. They argued that at the time of extirpation the nose primordium was not yet underlain by prospective forebrain, but they did not consider it significant that these two primordia, at this stage, practically touch each other in a tangential direction (see the fate map of Carpenter, 1937). In still other experiments Zwilling attributed the appearance of nose to induction by head mesoderm, yet these studies furnished only one case in support of this view. In this case, prechordal mesoderm implanted into the blastocoel brought forth a nasal canal and

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The work described is a revision of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Department of Biology, The University of Rochester, Rochester, New York.

[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 120–38, June 1956]

pit in the overlying belly ectoderm of the host in the absence of true brain though not of ganglionic and other tissues. In experiments by Emerson (1945) when various regions of late gastrula ectoderm were explanted, it was found that ectoderm alone could form nose, but when the underlying mesoderm had been included in the explants, olfactory differentiation was more frequent and more typical; however, all explants of both types which exhibited nose formation also contained brain fragments.

There are, on the other hand, numerous and more persuasive data which indicate that the forebrain primordium, after it has been induced by the head mesoderm, plays a determinative role in the emergence of the olfactory placodes.

Only in passing, reference may be made to the conditions in microcephalic animals which, as is well known, can be produced by a great diversity of chemical or physical interferences with early embryogenesis. There it was found that monorhiny always occurs associated with a reduced forebrain, and total absence of nasal tissue with still larger brain defects. However, if one were to conclude from these data that the forebrain inductively controls nose formation, this view would be no more defensible than the alternative assumption that brain as well as nose arise simultaneously under the inductive influence of head mesoderm. More convincing evidence in support of the notion that forebrain induces nose may be derived from the experiments with dead tissues of all sorts which, when grafted into the blastocoel, frequently induced brain and nose on the ventral side of the host (Holtfreter, 1934 *a*, *b*; Chuang, 1939; Toivonen, 1940). Contrary to the occasional induction of free lenses by such grafts we do not know of a single clear-cut case in which nose was induced without an adjacent forebrain. In these instances no head mesoderm was involved although it is possible, if improbable, that the noses were induced directly by agents coming from the dead graft rather than by the brain which was induced first. An intervention of mesoderm was furthermore excluded in experiments in which explants of gastrula ectoderm were briefly subjected to an injurious environment. Many of the explants differentiated into a multiplicity of brain diverticula and it was a striking feature that, in close contact with the neural tissue, the overlying ectoderm formed at least as many nasal organs as there were brain diverticula (Holtfreter, 1944; Yamada, 1950; Hayashi, 1955). The following observations dealing with more normal conditions lead to the same conclusion.

After transplantation of the head neural fold to the prospective gill region of a neurula the adjacent host ectoderm formed a nasal organ in contact with a telencephalic hemisphere which differentiated from the graft (Raven, 1935). Kucherova (1945) found that prospective forebrain grafted beneath the flank ectoderm of early neurulae induced the formation of nasal organs. Explants from early neurulae containing prospective forebrain and flank ectoderm exhibited brain and nasal organs, whereas the same flank ectoderm combined merely with head mesoderm failed to show the presence of a nose (Lopashov, 1937). In this connexion we may also mention the studies of Nieuwkoop (1950)

who found that a large piece of gastrula ectoderm placed into the neural plate becomes induced to differentiate into forebrain, the latter being frequently associated with nasal pits, again in the absence of head mesoderm.

Thus, in the search for the tissues which normally induce the nasal pits, much evidence has been accumulated in favour of the prospective forebrain and some weak evidence in favour of the rostral mesoderm. This evaluation of the data at hand differs somewhat from the balance-sheet which Yntema (1955) has drawn in his recent review on nose formation. Nevertheless, the data quoted above need not be contradictory. Both neural tissue and mesoderm may very well be engaged in nose induction, analogous to the synergistic action of those tissues in the case of ear induction (Yntema, 1950) or in other head structures. Since a clarification of this issue has not come forth, it was felt desirable to investigate it further.

MATERIALS AND METHODS

The eggs of *Amblystoma punctatum* used were collected from breeding ponds near St. Louis, Missouri, Hanover, New Hampshire, and Rochester, New York. Development of all embryos was retarded by temperatures of 5° to 12° C. in order to obtain embryos of the stages desired. The embryonic stages referred to in this paper are those of Harrison (see Hamburger, 1942).

The experiments were carried out under aseptic conditions according to the method described by Townes & Holtfreter (1955). After healing, the operated embryos were transferred to 10 per cent. Holtfreter's solution and allowed to develop for 10 to 12 days, at laboratory temperatures, during which time control embryos reached stage 41.

The present analysis involves isolation, defect, transplantation, and explantation experiments. In the transplantation experiments either the host or the donor was first stained with Nile blue sulphate. These animals were thoroughly washed in tap-water for 18 to 24 hours in order to eliminate the possibility of diffusion of the unbound stain into the non-stained tissues surrounding the transplant. To preserve the stain in sections the animals were fixed and dehydrated according to the method of Yamada (1937). The remaining experimental animals and controls were fixed in Bouin's solution, embedded in paraffin following the amyl acetate technique of Drury (1941), and stained in Delafield's hematoxylin and eosin. All data dealt with below are based on the study of histological sections.

Because of the close spatial relationship between nasal primordium and presumptive telencephalon (Carpenter, 1937) it is difficult to separate these two primoria surgically at neurula stages. Great care was taken to remove all the presumptive nasal tissue when attempting to extirpate it, and to leave it intact when extirpating the presumptive forebrain. To accomplish this, a cut was made through the ectoderm of the neural fold along the line shown by Hörstadius & Sellman (1945) to be the boundary between presumptive telencephalon and head epidermis. Since this boundary is not actually demarcated, it is hardly avoidable

that some of the cases inadvertently contained brain tissue when only prospective nose was thought to have been extirpated, or that the intended complete removal of the forebrain was not always achieved.

In the experiments in which the head mesoderm was removed it was attempted to limit the excised area to the foremost part of the prechordal material which underlies the anterior part of the neural plate and neural folds. This material comprises part of the anterior fourth of the archenteron roof which, according to Mangold (1933), differentiates into mesenchyme, endoderm, and head-muscle. In our experiments no muscle differentiation was observed. The terms nasal or olfactory organ or nose will be applied to typical structures containing a sensory epithelial mass plus nasal pit, while 'nasal mass' refers to less typical structures lacking a pit.

RESULTS

A. *Time of determination of the olfactory organ*

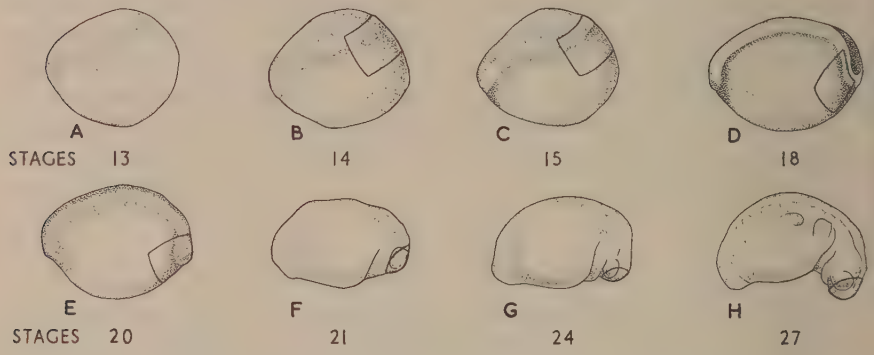
The previous work of Carpenter (1937) indicated that in *Amblystoma punctatum* the prospective nasal ectoderm can self-differentiate when transplanted to other head regions as early as stage 13. The following experiments were designed to test this capability over a wide range of stages by the use of three different methods. According to our results determination of the olfactory epithelium occurs much later than at stage 13.

1. *Isolation experiments.* A unilateral ectodermal area, including far more than one of the nasal organ primordia, was extirpated at stages 14 to 27. The areas used here and in some of the subsequent experiments are outlined in Text-fig. 1, B–H. After they had rounded into a ball the isolates were transferred from full strength to 10 per cent. Holtfreter's solution and cultured for a period that allowed the controls to develop to about stage 40 (10–13 days), then fixed and studied in sections. The upper part of Table 1 shows that essentially similar results were obtained from isolations performed at all stages before 21. Here nasal organs developed solely in those isolates which contained neural tissue that had accidentally been included in the explants. All the remaining isolates of these series lacked neural tissue and differentiated into unspecific balls of epidermis. On the other hand, a rather large number of the isolates from stages 21 to 27 could form nasal organs in the absence of neural tissue.

The capability for self-differentiation of the nasal primordium increased gradually with the time of isolation. At stage 21 only 3 out of 15 cases, at stages 22–25, 6 out of 15, and at stages 26–27, all of the 7 experimental cases formed nasal differentiations in the absence of neural and mesodermal tissues. Furthermore, with regard to organological perfection, the olfactory differentiations observed in explants from earlier stages were always inferior to those in explants from later stages. A comparison in the Plate of figs. A and B shows the difference between nasal structures in explants at stages 22–25 and 26–27 respectively. The degree of organotypical perfection of the olfactory organ was evidently

controlled by the presence of even small bits of forebrain tissue indicating a continued influence of the latter upon the shaping of the induced nasal primordium.

2. *Heterotopic transplantation experiments.* The isolations were supplemented by transplantation experiments in which the same areas as indicated in



TEXT-FIG. 1. Operational scheme showing the ectodermal areas which were isolated, transplanted, or simply removed, as projected upon Harrison stages 13 to 27.

TABLE 1

Results obtained from isolations and heterotopic transplantations of nasal primordia from various developmental stages

Operational stages:	14	15–16	17–18	19–20	21	22–25	26–27
				Isolates			
Number of cases . . .	29	45	50	24	15	15	7
Nasal organ and brain present .	5	2	3	1	0	1	0
Nasal organ present, brain absent	0	0	0	0	3	6	7
				Transplants			
Number of cases . . .	0	32	21	36	14	19	2
Nasal organ and brain present .	—	11	9	16	7	7	0
Nasal organ present, brain absent	—	0	0	0	4	11	2

Text-fig. 1, C–H were employed. The donors of the grafts, previously stained with Nile blue, ranged from stages 15 to 27. The head ectoderm was grafted to the anterior flank region of non-stained hosts of the same or somewhat younger stages. While comparing the data in the upper half with those of the lower half of Table 1, note that the transplants gave much the same results as the comparable isolates. Transplants from stages below 21 produced olfactory differentiation only when neural tissue had been transplanted along with the nose anlage, whereas transplants from stages beyond 20 could form a nose in the absence of neural tissue (Plate, fig. C). Perhaps due to the more favourable environmental

conditions in the host the transplants formed relatively larger and better differentiated nasal organs than did the corresponding isolates.

The data obtained from both transplantation and isolation experiments indicate that the nasal primordium is not determined before stage 21. Its determination seems to be caused by forebrain tissue since nose ectoderm isolated before stage 21 developed olfactory structures only in association with an adjacent brain fragment while no head mesoderm was present. At subsequent stages the prospective nose ectoderm could self-differentiate in the absence of adjacent brain tissue; it had become determined meanwhile.

TABLE 2

Results obtained after unilateral extirpation of the nasal primordium at various stages

Operational stage	*Number of cases*	*Nose absent*	*Nose present*	*Approximate size of nose present on operated side*			
				Normal	*Slightly smaller*	*Three-quarters*	*One-quarter*
14 . . .	29	2	27	18	5	4	0
15–16 . .	41	10	31	12	6	10	3
17–18 . .	51	14	37	0	13	12	12
19–20 . .	32	17	15	0	1	13	1
21 . . .	36	19	17	0	0	6	11
22–25 . .	28	10	18	0	2	2	14
26–27 . .	40	16	24	0	2	7	15

3. *Unilateral excision of a nasal primordium.* Additional evidence in support of the above conclusions was derived from experiments in which unilateral extirpations of the nasal primordium were performed on embryos at stages 14 to 27 (Text-fig. 1, B–H). The surrounding ectoderm was allowed to heal over the wound and the animals were cultured until they reached stages 40 to 41. In the histological examination of the sectioned material the unoperated side served as the control for estimating the normality of any nasal structure which might appear on the operated side.

It came as a surprise that in spite of the removal of such a large piece of ectoderm, including all of the nasal primordium, the operated side formed a more or less normal nose in many cases. This differentiation must have arisen from the surrounding ectoderm which shifted over the wound. It means that the head ectoderm far away from the nasal primordium is capable of reacting to the inductive stimuli of the nose region.

The details presented in Table 2 furnish the following general picture. The capability of the healed-over head ectoderm to give rise to at least a defective nose is present in all of the stages investigated. However, this capability definitely decreases with age, both with respect to statistical frequency and histological perfection of the noses formed. One may ascribe this relationship between stage

and positive results to a progressive loss of competence of the head ectoderm to respond to nose-inductors, a conclusion which fits well into the general conception of the age-conditioned ectodermal competences. But when looking at the results more closely one observes deviations from this general picture which we shall now attempt to account for.

One might wonder, for instance, why noses developed in the healed-over head ectoderm only in a certain percentage of cases in the experiments with early stages (14–20) before the nasal primordium is determined. Histological examination showed that this apparent lack of response to nose-inductors can be related to unavoidable variables in the technical procedure of making these defects. It was found that when the operated side showed absence or defects of the nose, the following abnormalities were usually associated with it: absence or defects of the eye, absence or extensive reduction of the cerebral hemisphere on the operated side. Thus the data which seemingly fall out of line rather support the notion that the presence of neural structures is necessary for the induction of a nose. They do not reflect upon the competence of the head ectoderm.

Such abnormalities of the brain-eye system did not occur in embryos operated on beyond stage 20 because here the experimenter could easily distinguish prospective neural tissue from prospective nasal ectoderm. The abnormal nose differentiations turning up in the experiments with these advanced operational stages could not be correlated with any defects in the inductor system. They were obviously due to stage-conditioned changes of competence of the adjacent head ectoderm which shifted over the region of nose-inductors.

From these results one may conclude that nose-inducing stimuli are operative as late as stage 27 and that the ectoderm can react to them up to this stage, although the reaction of the heterotopic head ectoderm becomes progressively less pronounced with increasing age.

B. *Capability of heterotopic ectoderm to react to nose-inducing stimuli*

The competence for nose differentiation extends, at early stages, over a wide area of the head ectoderm (in the early gastrula over the entire ectoderm) and it seems to become locally restricted at more advanced stages. In order to test whether ectoderm entirely outside the head region is also capable of reacting to nose inductors, the following experiments were performed.

Anterior flank ectoderm from non-stained embryos of stages 15–16, 19–20, and 22–25 was transplanted in place of the extirpated prospective nasal ectoderm of vitally stained embryos of the same stages. In no instance was the graft capable of forming an olfactory structure. Many cases among all operational stages showed complete absence of a nasal organ while others exhibited noses within the healed-over ectoderm of the host. The results, in addition to demonstrating that the grafted flank ectoderm is incapable of response, lend further support to the conclusion drawn above that head ectoderm is able to react to nose-inducing stimuli. The summary of Table 3 shows that the results fall into three rather

well-defined groups with regard to the condition of the olfactory region on the operated side.

1. *Olfactory organ absent on the operated side.* In all cases exhibiting absence of a nasal organ it was noted that the grafted ectoderm fully covered the olfactory area and prevented the surrounding head ectoderm from healing over the nasal area. The largest percentage of such cases occurred in embryos operated on at stages 22 to 25. The failure of progressively older stages to produce a nose may

TABLE 3

Results obtained after replacing the extirpated nasal primordium by anterior flank ectoderm. Hosts and donors were of the same stage. The nasal organs observed on the operated side were always formed by healed-over host ectoderm

Operational stages	Number of cases	Appearance of olfactory structures on the operated side		
		Absent	Normal	Small, formed at juncture of graft and host tissues
15–16 . . .	18	4	2	12
19–20 . . .	22	4	1	17
22–25 . . .	15	12	0	3

be ascribed partly to changes of competence of the head ectoderm and partly to the fact that at the later stages the graft could be placed more exactly over the entire nose area.

2. *Large nasal organ formed by host ectoderm.* This occurred when the grafted flank ectoderm did not cover the whole area of the excised prospective nose ectoderm. A nose developed at the normal site.

3. *Incomplete nose differentiations.* In some instances nasal pits arose close to or within the boundary line between host and grafted ectoderm, always involving merely the ectoderm of the host. In two transplantations made at stage 19–20, host ectoderm produced a nasal mass quite outside the normal nose area, an observation which might suggest that nose-inductors are more widely spread than is indicated by most of the data showing the appearance of the experimentally induced nose at the proper site.

The experiments of this chapter actually test the nose-competence of both the flank and head ectoderm. The data show that flank ectoderm from stage 15 and beyond can no longer respond to nose inductors but becomes epidermis. Due to variations in the size and location of the transplants, the wound in the nose region frequently became partly or even entirely covered by healing head ectoderm which then formed a fragmentary or normal nose, thus confirming the results obtained from the defect experiments summarized in Table 2.

C. *Inductive capability of the frontal head region at various stages*

The preceding data show that head ectoderm, normally not destined to become a nose, can produce it after shifting over the nose region. Evidently some sub-

ectodermal tissues of the frontal region possess nose-inducing capacity between stages 15 and 27. In order to study more fully a possible relationship between developmental stage and inductive capacity and at the same time test the nose-competence of flank ectoderm of a stage earlier than those used before, the following experiments were made.

The same areas of vitally stained prospective nose ectoderm as in the preceding experiments and as illustrated in Text-fig. 1, were excised at stages 15 to 27 and then replaced by a non-stained graft of anterior flank ectoderm which was

TABLE 4

Results obtained after transplantation of flank ectoderm from stage 13 embryos in place of the extirpated nasal primordium of embryos at later stages

Operational stages	*Number of cases*	*Normal nose induced in the graft*	*Nose absent*	*Small host nose at juncture of graft and host ectoderm*
15–16 . . .	15	10	1	4
17–18 . . .	9	4	2	3
19–20 . . .	9	5	0	4
21	17	14	0	3
22–25 . . .	9	3	4	2
26–27 . . .	6	2	2	2

always taken from an early neurula not yet showing signs of neural folds (stage 13, Text-fig. 1, A). Unlike the flank ectoderm of stages 15 to 27, the present grafts proved to be well competent to react to nose inductors. Let us discuss the results as listed in Table 4.

It may be pointed out in the first place that in confirmation of the preceding results all stages between 15 and 27 exhibited nose-inducing activity. Secondly, the noses observed had usually been derived from the graft (Plate, fig. D), and not, as before, by the healed-over head ectoderm; sometimes they were composed partly of grafted and partly of host-ectoderm. Some apparent exceptions and certain details may be discussed.

In a few cases when a nose was entirely absent from the operated side, the defects were always accompanied by defects of the cerebral hemisphere on that side. In the remaining cases it was obviously a question of the topographical location of the graft which decided whether the nose was formed by the graft, the host, or both. In cases in which the nose originated entirely from the graft, the latter covered more than the nose region. In other instances when graft and host ectoderm had competed in an attempt to cover the wound, both tended to contribute to the differentiation of a nasal organ at the proper site. In some cases, the nasal organ consisted partly of host and partly of grafted ectoderm. There was quite a variation in the size and perfection of the newly induced nasal organ.

These experiments with early neurula ectoderm brought up a complication. In some instances the grafted flank ectoderm from stage 13 did not merely become

induced to form nasal epithelium, but formed a forebrain as well. This phenomenon belongs to the category of homoiogenetic induction previously investigated by the use of gastrula ectoderm (Mangold, 1929; Mangold & Spemann, 1927; Holtfreter, 1938). There is no way of telling whether the noses observed in such situations have been invoked directly by the host tissues or indirectly by the forebrain which was first induced in the graft. The latter interpretation seems to be the more likely one. Brain plus nose inductions were observed in only 4 out of 38 cases in the present experiments. An example is given in Fig. E of the Plate, which deals with an embryo operated at stage 26–27. The section shows a normal nose on the unoperated side (N_1) and another on the operated side (N_2), the latter lying quite dorsally. This instance suggests once more that nose inductors extend beyond the normal site into more latero-dorsal regions of the head. The same section also shows the reactions of the graft, namely a homoiogenetically induced brain vesicle (*IB*) closely associated with a nose (N_3), the latter apparently being called forth by the former. A similar condition is illustrated by Fig. F of the Plate with the slight difference that here the graft produced a nose (*N*) at the normal site, again apparently induced in response to stimuli from forebrain (*IB*) which was induced first.

Evidently the ectoderm of stage 13 is on the verge of changing its competence for brain differentiation to that of nose differentiation. At any rate, the anterior flank ectoderm of stage 13, contrary to that of stage 15, is fully competent to react to nose-inducing stimuli. The observation that stages as late as 27 could induce nose in grafts is in agreement with our preceding data.

D. *Attempts to histologically localize the nose inductors*

It has become clear by now that some subectodermal tissues of the nose region, over a wide range of stages, emit nose-inducing stimuli which apparently extend somewhat beyond that region where they normally become manifest. Several of the above observations indicated that such stimuli stem from the forebrain primordium, but they did not quite exclude the possibility that in normal development the underlying mesoderm may also play an inductive role. The question has been examined further in a number of defect and explantation experiments in which (1) prospective nose plus adjacent forebrain were unilaterally excised, and (2) these excised portions were cultured as explants. The conditions obtained in the defective embryos were compared with those in the corresponding explants. Let us consider the data of this double-faced experiment simultaneously (Table 5).

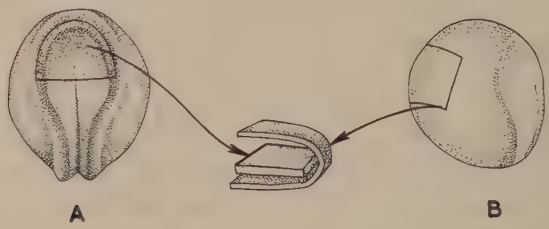
1. *Removal of prospective forebrain plus nasal ectoderm.* At all stages used (15–25), complete failure of nose differentiation on the operated side was observed in the majority of cases. In accordance with what has been pointed out above, absence of a nose was found to be correlated with absence or extreme size reduction of the cerebral hemisphere on the same side. In the other cases listed in Table 5 when a nose was present the unilateral excision of the prospective

forebrain had not been successful and the noses appeared in close contact with often voluminous parts of forebrain which had either been left behind or had possibly regenerated.

TABLE 5

Data obtained after unilateral extirpation and explantation of presumptive forebrain plus nasal primordium at various stages

Operational stages	Defect experiments			Explants	
	Number of cases	One nose absent	Two noses present	Number of cases	Brain and nose present
15–16 . .	16	14	2	6	6
17–18 . .	15	13	2	15	10
19–20 . .	23	22	1	10	7
21 . . .	39	38	1	11	11
22–25 . .	15	13	2	3	3



TEXT-FIG. 2. Operational scheme showing the area of medullary plate and fold (A) which were excised and explanted together with anterior flank ectoderm from an embryo at stage 13 (B).

The corresponding explants developed, with a few exceptions, into an epidermal ball containing a large well-differentiated brain portion in contact with an olfactory organ. In 8 out of 45 explants no nasal differentiation was observed, but the amount of neural tissue was extremely small. The data leave no doubt that the prospective forebrain emits nose-inducing stimuli.

2. *Extirpation of prospective forebrain and underlying mesoderm, separately or together.* It should be realized that the solution of quite a few embryological problems depends upon the unerring skill of the experimenter in making operations on the embryo precisely as planned. We do not pretend to have achieved this technical perfection. Nevertheless, great care was taken to separate the primordia in question as cleanly as possible in the absence of well-defined landmarks. The procedure was as follows.

In the first experimental series (*a*) the anterior quarter of the medullary plate including the transverse neural fold (stage 15) was removed according to the scheme of Text-fig. 2. In another series (*b*) the anterior neural plate was partly cut out, flapped back, and the most anterior one-seventh of the archenteron roof

was excised. The neural plate was returned to its former position where it healed normally. In series (*c*) the same medullary area together with the underlying archenteron roof was removed.

(*a*) *Embryos with a defective forebrain.* Of the 34 cases studied in this series, 14 developed two, 14 only one, and 6 no nasal organs. As to the first group, the presence of two noses was, in 12 cases, associated with the presence of two cerebral hemispheres of more or less reduced size. In 2 cases a single, large, medially located forebrain was present, in contact with a very large nasal mass having two pits (synrhiny). In the second group, the appearance of a single nose was always concomitant with the presence of a single hemisphere. The latter was either situated laterally, disconnected with the posterior brain parts (8 cases), or it represented a more medially located vesicle bulging out laterally towards the adjacent nose (6 cases). Finally, in the third group, all 6 cases showing absence of both noses also showed complete absence of a forebrain.

(*b*) *Embryos with defects in the rostral archenteron roof.* In all 42 cases in which the rostral portion of the archenteron roof had been excised, the animals developed two noses. In 23 cases the noses appeared at approximately the normal site, each nose associated with a well-defined cerebral hemisphere or the lateral walls of a single large forebrain. In 19 cases the olfactory structures were medially situated, fused to a varying extent, and exhibited accordingly one or two pits. In the latter instances the telencephalic tissue represented a single, rather small medial vesicle. Thus the removal of the rostral part of the archenteron roof did have some effect upon the bilateral distribution of the forebrain material but it failed to produce complete absence of nose. The subnormal noses were always correlated with subnormal brains.

(*c*) *Embryos with defects in forebrain and subjacent archenteron roof.* Rather unexpectedly, out of 33 embryos subjected to operations of this kind, 22 developed two noses, 8 a single nose, and only 3 showed no nose. This wide variation in the results can be understood only if one takes into account the technical difficulties involved in such surgical experiments and the possibility that fragments of medullary plate or mesodermal tissues, inadvertently left behind in the operated embryo, may have undergone regulation or reconstitution. Let us examine these three groups of results more closely.

Obviously due to an incomplete excision of the prospective forebrain, 16 cases out of 33 developed two slightly reduced telencephalic diverticula associated with two nasal organs in the normal position, while in 6 cases a single, small, medial brain vesicle associated with synrhinic formations was observed. In 8 cases, when only one nose appeared, a lateral portion of the prospective forebrain had been left behind in the embryo. In the remaining 3 cases when no nose developed at all, no forebrain was formed either.

Thus we find a close correlation between presence, absence, and completeness of forebrain structures, on the one hand, and corresponding degrees of bilaterality and perfection of nose differentiation, on the other. The argument that this

parallelism may be caused by a common factor, namely defects in the archenteron roof, which may simultaneously have induced both forebrain and nose, is not borne out by the present experimental data. It appears that if in consequence of excision of the cephalic archenteron roof only one nasal organ was formed, this was due to a defect in forebrain differentiation and not directly to reduction of the head mesoderm.

In this connexion one may wonder why the excision of the rostral archenteron roof did not produce the synopthalmic or cyclopic larvae as recorded by Adelmann (1937) and Mangold (1931), but at most merely a single forebrain vesicle associated with synrhiny. This apparent discrepancy seems to be explainable by the fact that in the present experiments the excisions of the archenteron did not reach as far back as in those of the workers mentioned above.

3. *Combination of excised presumptive forebrain and/or underlying archenteron roof with flank ectoderm in explants.* In further attempts at localizing the nose inductors, either (*a*) an anterior part of the medullary plate, or (*b*) its underlying endo-mesoderm, or (*c*) both were isolated at stage 15 and placed into a jacket of ectoderm. The ectoderm quickly shifted over the enclosed tissues. Such explants were cultured for about 12 days.

(*a*) For technical reasons, only one lateral half of the medullary region outlined in Text-fig. 2A was used in this series of experiments. The donors of the medullary plate had been previously stained with Nile blue. Of the 41 explants studied histologically, 10 differentiated a nose in the non-stained ectoderm. The brain tissue was always in close association with the induced nose and no other tissues were present in these explants (Plate, fig. G). In the other 23 cases the explants contained some mesenchyme which had been derived either from the neural crest of the transverse neural fold (Hörstadius & Sellman, 1945) or, less likely, from mesodermal cells of the archenteron roof which had defeated our endeavour of cleaning the excised medullary tissue from any adjacent cells. The presence of mesenchyme significantly influenced the results. In two explants of this kind a nose appeared close to the neural tissue in spite of the presence of mesenchyme. In the other 21 cases a mantle of mesenchyme separated brain tissue from the ectodermal covering and no nose was induced. Clearly, the mesenchyme inhibited rather than promoted nose induction.

(*b*) The endo-mesoderm underlying the anterior portion of the neural plate and nose primordium was excised from stage 15 and placed into an envelope of competent flank ectoderm. In this crucial experiment none of the 20 explants studied showed nose formation.

(*c*) The combination of excised presumptive forebrain plus underlying archenteron roof with flank ectoderm gave the following results. Out of the 35 explants studied only 4 developed a nose in the ectodermal covering (Plate, fig. H). There was intimate contact between brain and nose tissues. In the remaining cases the explants became more or less vesicular due to the differentiation of mesenchyme which appeared between the centrally located brain

fragment and the epidermal envelope. No other tissues, such as head-muscle or cartilage, ever developed in the explants. In these 31 cases no nose was induced.

To these data may be added those of another, somewhat different, experiment. There, the nose primordium from one side of a neurula (stage 15) was isolated together with the subjacent rostral endo-mesoderm. In 20 of such explants studied, no nose differentiation was observed.

These results seem to demonstrate that the presumptive forebrain and not the rostral head mesoderm is responsible for the emergence of the olfactory organs.

DISCUSSION

Capability of self-differentiation of the presumptive nose in successive stages of development

The observations of isolates and heterotopic grafts of the nasal primordium at various stages indicate that the nose is not determined before stage 21 (*Amblystoma punctatum*). Experiments performed on successively older stages (21–27) revealed a general increase of the capability for self-differentiation of the primordium.

These data differ from those of Carpenter (1937) on *A. punctatum*, and Zwilling (1940) on *Rana pipiens*. Carpenter grafted nose anlage into a lateral head region of embryos at stages 13–27 and observed nose differentiation in 18 cases. In only one of these cases (stage 14) did a nasal organ occur in the absence of transplanted brain tissue, but the microphotograph of this case (fig. 13) shows the nose in the proximity of the host's nasal organ, suggesting that it arose due to nose-inducing stimuli of the host and not by self-differentiation of the graft. In *R. pipiens*, on the other hand, the nose actually appears to be determined before the rising of the neural folds, because in xenoplastic transplants from stage 14, a nose differentiated in 8 out of 15 cases, while no brain was observed in the graft (Zwilling, 1940). Evidently the nasal organ in *A. punctatum* is determined much later than it is in *R. pipiens*. Such species-specific differences in the time of determination are well known in the development of other organs, e.g. the lens.

Competence of the ectoderm outside the nasal primordium to react to nose-inducing stimuli

Despite unilateral excision of the nose primordium together with a large area of lateral head ectoderm, nose defects failed to appear in many cases (stages 14–27). This means that the head ectoderm destined to form epidermis, lens, or balancer is not yet fixed in its fate and can react to the nose-inducing stimuli of the frontal region after it has shifted over the wound. These results agree with those of Carpenter (1937) who observed a nose in the normal position after replacing the nasal primordium by heterotopic head ectoderm at stages 14 to 26.

It is well known from the study of diverse head structures that the competence of the ectoderm changes both with age and with regard to embryonic region. The

present studies extend this notion to the potentiality for nose formation. At the preneurula stage apparently any part of the ectoderm can differentiate a nose when properly stimulated. Here we find that at the early neurula stage (13) the anterior flank ectoderm is still capable of this reaction, but that from stage 15 onwards the capability is lost. However, the head ectoderm outside the nose primordium retains nose-competence at least until stage 27. The fact that the noses formed by head ectoderm become less frequent and smaller with increasing age of the embryo operated on suggests that within the head ectoderm nose competence becomes more and more limited to the region of the nose primordium.

There were a few instances in which grafted flank ectoderm produced not only a nose but brain as well. Such complex inductions are known to occur following the implantation of anterior medullary plate or its subjacent tissues into a gastrula (Mangold, 1929; Waechter, 1953) and, in reciprocal experiments, when gastrula ectoderm is grafted into the lateral head region of a neurula (Holtfreter, 1933). In the present experiments brain inductions were elicited only in the youngest ectoderm used (stage 13). These cases illustrate once more the age-conditioned change of competence of the ectoderm and at the same time demonstrate that hosts up to stage 27 can still induce accessory brain fragments. The noses which differentiated together with the accessory brain portions were probably induced secondarily by the latter, rather than directly by host tissues.

Period of inductivity of the frontal region

The capability of subectodermal tissues of the nasal region to induce nose in healed-over head ectoderm or in grafted flank ectoderm is present as late as stage 27, the latest stage studied here. These data correspond to those of Kucherova (1945) who observed nose inductions in flank ectoderm grafted from early neurulae to the head region of tail bud stages of *Rana esculenta* and *Bufo viridis*.

In some cases a nose occurred in the dorso-lateral head region, far from the normal site, suggesting that nose-inducing stimuli extend over a larger area than is indicated by normal development. Zwilling (1940), on the basis of similar observations, arrived at the same conclusion.

Which tissue induces a nose: prospective forebrain, head mesoderm, or both?

Critical evaluation of the data of previous workers, concerning the factors involved in nose induction, leads to the supposition that prospective forebrain rather than head mesoderm is of dominant if not sole importance in this process. However, many of these observations, quoted in the introduction of this paper, seem to lack conclusiveness. The data collected in the present investigation provide ample support for the view that the head mesoderm not only lacks nose-inducing ability, but that it seems to interfere with rather than to promote nose induction in explants. Let us briefly list the data pertinent to this question.

Explants from a neurula containing only prospective forebrain and adjacent rostral ectoderm developed a nose in the majority of cases, whereas isolates

composed of the nose primordium plus the rostral mesoderm or of rostral mesoderm and competent flank ectoderm failed to do so. Mangold (1933) and Hama (1949) already observed that even much larger portions of the prechordal mesoderm than used here cause no or mainly weak inductions when confronted with gastrula ectoderm.

The defect and transplantation experiments gave results along the same line. Whenever the removal of the whole forebrain primordium had been successful, no nose developed (p. 131). Removal of the tissues underlying the head plate did produce subnormal development of the forebrain, but there was no way to decide whether or not the concomitant mono- or synrhinic formations were caused directly by the operation or indirectly by the subnormal development, and hence, the reduced inductive power of the forebrain. This argument applies to similar situations in which defects in the archenteron roof became expressed not only in the primarily but also in the secondarily induced structures (see microcephaly, p. 121).

To conclude then, there is no case showing that head mesoderm alone can induce a nose. On the contrary, the presence of mesenchyme, derived from either the head mesoderm or from the transverse fold, tended to prevent nose induction by forebrain tissue.

The above results are in accord with those of Lopashov (1937) and Kucherova (1945) who found in isolation and transplantation experiments that parts of forebrain alone can induce a nose, but they throw doubts upon the conclusiveness of the cases presented by Carpenter (1937) and Zwilling (1940) which seemed to speak in favour of a nose-inducing action of the head mesoderm. As mentioned before, the data of the latter authors fail to show that a nose can be induced in the complete absence of neural tissue. A single case of Zwilling (1940) demonstrated the differentiation of a nasal canal and pit in the absence of brain but, nevertheless, showed neural tissue in the form of a 'ganglion-like mass'.

Thus the mechanism of nose induction appears to differ from that of otocyst induction which involves the determinative actions of both mesoderm and brain tissues (Yntema, 1950). According to Cooper (1943), at much later stages mesenchyme (mainly of neural crest origin) does assist in the shaping of the olfactory organs. This influence is probably of a predominantly mechanical and not inductive nature.

SUMMARY

1. This study deals with the tissue factors engaged in the induction of the nasal organs in embryos of *Amblystoma punctatum*. Isolations and heterotopic transplantations of a large area of head ectoderm, including all the nose primordium of one side, demonstrate that the nose primordium is not determined before stage 21.

2. By unilaterally excising nasal primordia, and either allowing the surrounding head ectoderm to heal over the wound, or replacing the excised primordium

with anterior flank ectoderm from embryos at stages 13 to 25, it was demonstrated: (1) that heterotopic head ectoderm of all the stages studied is competent for nose formation, (2) that anterior flank ectoderm from stage 13 is able to react to nose-inducing stimuli, while (3) anterior flank ectoderm from stages beyond 14 no longer has this ability. The nose competence of heterotopic head ectoderm progressively decreases with increasing age.

3. Transplantations of competent ectoderm in place of the excised nasal primordium at stages 15 to 27 show that nose-inducing capabilities are retained within the nasal region until the latest stages studied here. In some instances, early flank ectoderm, grafted into the nose region, was induced to form a brain fragment associated with a nasal organ. The latter was evidently induced secondarily by the former.

4. Isolates of the prospective nasal ectoderm, previous to stage 21, differentiate a nose only if part of the adjacent forebrain anlage is included in the explants.

5. Removal of the presumptive forebrain, with or without the underlying archenteron roof, could result in (1) complete absence of noses if the telencephalon is absent, (2) absence of one nose if the cerebral hemisphere is absent or very small, or (3) synrhiny or monorhiny if the forebrain is a small medial vesicle. The removal of the anterior one-seventh of the archenteron roof alone did not directly affect the number of noses present but tended to produce hypomorphic forebrains which in turn were associated with subnormal nose formations.

6. The capability of prospective forebrain and the most anterior portion of the archenteron roof to induce nose was tested by wrapping these tissues, separately or together, in competent ectoderm. Nose inductions occurred only in those explants which contained brain, whether mesoderm was present or not. On the other hand, ectodermal explants enclosing head mesoderm alone showed no inductions. The frequency of nose inductions in explants containing presumptive forebrain was much higher than in those with mesoderm in addition to brain. Mesenchyme seemed to prevent rather than promote nose induction.

The data taken together support the view that inductive stimuli emanating from the prospective forebrain are solely responsible for the emergence of the olfactory organs.

ACKNOWLEDGEMENT

The author wishes to express his appreciation to Professor J. Holtfreter who suggested and supervised the research programme.

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EXPLANATION OF PLATE

Fig. A. Nose differentiation (*N*) in an explant of rostral head ectoderm isolated at stage 22–25.

Fig. B. Nose differentiation in an explant of rostral head ectoderm isolated at stage 26–27.

Fig. C. Nose differentiation (*N*) in rostral head ectoderm from stage 22 transplanted to the latero-ventral flank region.

Fig. D. Section through the nose region of an embryo which has induced a nasal organ (*N*) in flank ectoderm transplanted at stage 13 to a host at stage 19–20.

Fig. E. Transplantation of anterior flank ectoderm at stage 13 to the nose region of a host at stage 26–27. The host formed two noses, one of which (N_2) is atypically situated. The nose formed by the graft (N_3) is closely associated with a secondarily induced brain fragment (*IB*).

Fig. F. The same experiment as illustrated by fig. E except that a younger host was used (stage 22). (*IB*) secondarily induced brain, (*N*) nose induced in graft.

Fig. G. Explant of vitally stained medullary plate and fold wrapped in non-stained flank ectoderm. Note the induced nose (*N*) at the lower left in contact with the forebrain.

Fig. H. An induced nose (*N*) can be seen at the lower right in contact with the forebrain where no mesenchyme intervenes.

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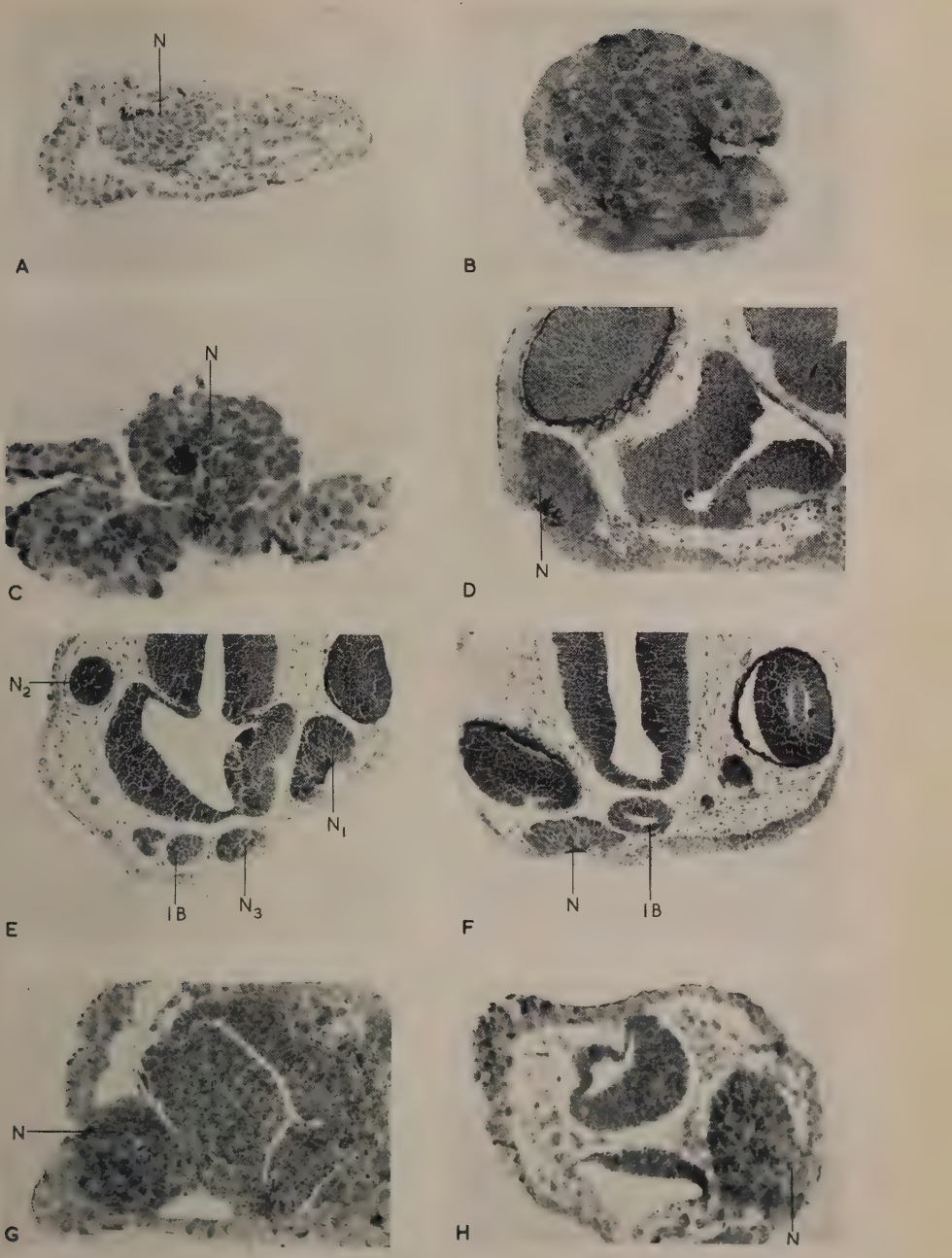
N
A
B
N
C
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N_2
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IB
N_3
E
N
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G
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Compensation in the Remaining Pronephros of *Triturus* after Unilateral Pronephrectomy

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WITH ONE PLATE

INTRODUCTION

As Howland (1916, 1921) originally showed and many other workers have confirmed, an amphibian larva can survive satisfactorily with one pronephros, but it cannot survive bilateral pronephrectomy (Swingle, 1919; Shimasaki, 1930*a*; and Cambar, 1944 *a*, *b*, 1948) unless, as shown by Cambar, the mesonephros has become well established and functional.

The remaining pronephros, when one has been removed, becomes noticeably larger in *Rana sylvatica* (Swingle, 1919), *R. nigromaculata* and *Bufo vulgaris japonicus* (Miura, 1930 *a*, *b*), *Rana dalmatina* and other Anura (Cambar, 1948), *Triton alpestris* (Machemer, 1929), and *Amblystoma punctatum* (Howland, 1916, 1921; Detwiler, 1918, p. 520). Hiller (1931) has also studied the problem in the latter species from the functional aspect in parabiotic twinning. However, except for the quantitative papers of Howland and Hiller, both of which appear to suffer from insufficiency of data, there is no information of quantitative nature.

The following account is a quantitative histological analysis of pronephric compensation. The results support Howland's conclusions that a remaining pronephros adjusts itself to the artificially created situation by compensating in a variety of ways.

MATERIAL AND METHODS

The larvae of *Triturus cristatus carnifex* (Spurway, 1953; Fox, 1955*a*) used in 1952 and 1953 were from the same breeding pair, ♀ 46 and ♂ 39, which had been presented to Dr. H. Spurway by Professor G. Montalenti of Naples in 1950. Only those larvae laid in the spring of 1953 were quantitatively analysed, but others of the 1952 crop were examined for general morphological features. Operations were performed at the early caudal bud stage (equivalent to Harrison stage 22–24 for *Amblystoma punctatum*) after decapsulation in full strength

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[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 139–151, June 1956]

Holtfreter solution. The pronephros in urodeles arises from nephrotome material beneath somites 3 and 4 (Field, 1891; Howland, 1921; Fales, 1935), and the duct from material beneath somites 5, 6, and possibly 7 (O'Connor, 1938; Holtfreter, 1944). Just posterior to the presumptive branchial region, the pronephric rudiment can be seen as a small protuberance bulging slightly from beneath the epidermis. It is closely associated with the presumptive fore-limb region (Harrison, 1915; Detwiler, 1918). Operations were made with sterilized glass needles (Hamburger, 1950). A small incision was made over the pronephric blastema, the epidermis was folded back, and the pronephric rudiment was visible as a small, whitish glistening mass. The latter was then removed, care being taken to damage the surrounding tissue as little as possible. Even so, in many cases, the operation damaged the fore-limb blastema, and some of the pronephrectomized specimens at fixation showed stunting or even absence of the fore-limb on the side of the operation. The embryos were allowed to remain in full-strength Holtfreter solution for 2 hours to facilitate healing, and then they were transferred to Holtfreter solution diluted ten times, containing 0·1 per cent. sodium sulphadiazine. The base of each crystallizing dish in which individual specimens were kept was covered with a thin layer of gelatin. Controls were treated in an identical manner except that instead of removing a pronephros on one side, a surface wound was made, but the pronephric blastema lying beneath the epidermis was undisturbed. In all fifteen controls (30 pronephroi) and ten pronephrectomized specimens (10 pronephroi) were statistically analysed. Members in each group were killed in Smith's fixative at two periods, the controls at 10 and 17 days after operation, the pronephrectomized at 10 and during a short period around 15 days after operation. Specimens were embedded in paraffin wax, sectioned transversely at 10 μ, stained with Ehrlich's haematoxylin, and counterstained with aqueous eosin. Other specimens were stained with Mallory and Heidenhain's azan stains. The following measurements of the pronephroi of the two groups were compared: (*a*) nuclear populations; (*b*) volume of the total mass of cells; (*c*) volume of the lumina of the tubules; (*d*) overall volume of the pronephros (volume of the mass of cells plus lumina volume); (*e*) internal surface area of the tubules; (*f*) anteroposterior length of the pronephros; (*g*) calculated volume of the individual pronephric cell.

The measurements of each individual specimen were related to its nose-to-cloacal length, the latter being used as an index of development. In the controls the two pronephroi were analysed and the mean of the particular measurement in question was taken. In order to obtain the nuclear population of the pronephros, the first 10 sections, then every other section, and, finally, the last 10 sections, were analysed. Every nucleus of the pronephric tubules seen in the section by focusing up and down was counted. Each section not counted was considered to be the average of the section in front and behind it. The method was the same as that used to investigate nuclear populations in the pronephros of *Triturus cristatus karelinii* (Fox, 1955*b*). Corrections to the crude counts were

applied to allow for nuclear length at right angles to the section thickness (Abercrombie, 1946). This nuclear length does not change during the range of development studied in either control or operated specimens; in both groups it is approximately 12 μ. In order to measure the volumes of the tubule cells and lumina, sections were microprojected at 480 diameters, and the areas of these components were measured by a planimeter. The first seven sections, then every fifth, and, finally, the last seven sections, were measured (amounting to approximately 40 per cent. of the pronephros). The measurements of the remaining sections were interpolated in the series. Internal surface area of the tubules in each section was obtained by measuring the internal length of line of the tubule walls with a map measurer. A check of the method of sampling amongst the sections was made in one control pronephros by comparing results from the usual method, in which 19 out of 43 sections (44 per cent.) were measured, with those from measurements on 31 sections (72 per cent.). Differences for the volumes of lumina were 3·8 per cent., volumes of tubule cells 3·2 per cent., and internal surface area 1·7 per cent. The sampling would thus seem reliable enough. The volume of the individual cell of the pronephros was found by dividing the total number of nuclei into the total volume of the tissue. This is justified, as binuclear cells are rare. Nose-to-cloacal length was obtained by summing the total number of transverse sections from the extreme anterior end to the region where the pronephric duct opens into the cloaca. The range of time studied was from 10 to 17 days after operation; total lengths of the larvae within this range were between 10 and 15 mm. approximately, and nose-to-cloacal length 5·5 to 7·5 mm. approximately.

RESULTS

(*a*) *Quantitative changes*

When the two control subgroups, killed at 10 and at 17 days after decapsulation and wounding, are compared with each other, the pronephroi show considerable differences (Table 1). The mean (of both the right and left pronephroi) of the nuclear population significantly increases by 23 per cent., volume of the lumina by 78 per cent., and total internal surface area of the tubules by 55 per cent. The mean total cell volume does not significantly change. As the total cell volume does not increase and the nuclear population does, the mean volume of the individual pronephric cell falls significantly, by about 14 per cent. As a result of the increased volume of the lumina and the constant total cell volume, there is a significant increase in the overall volume of the pronephros of 23 per cent. The mean antero-posterior length of the pronephros would appear to shorten slightly, but this change is not statistically significant. In terms of nose-to-cloacal length, these changes are shown by the regression lines in Text-figs. 1–7.

When the changes with time of the pronephrectomized specimens are analysed (Table 1) all the changes which take place in the controls between 10 and 17 days are seen. In addition, there are increases in the means of the total volume

of cells and the antero-posterior length of the pronephros. Again, the greatest increases are those of lumina volume (149 per cent.) and internal surface area (86 per cent.). Means of the nuclear population show an increase of about 38 per cent., overall volume of the pronephros of about 61 per cent., total volume

TABLE 1

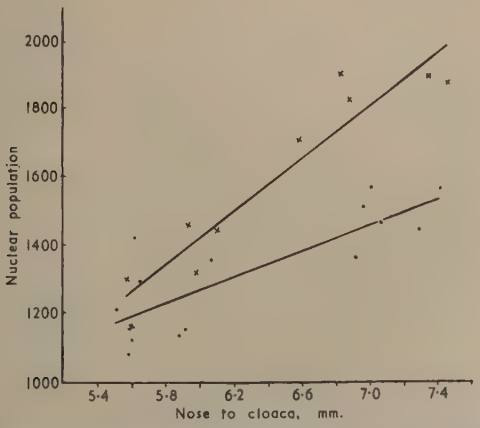
Means with standard errors of various measurements on the four groups of control and operated animals

	Controls	*Controls*	*Pronephrectomies*	*Pronephrectomies*
No. of specimens	9	6	5	5
No. of pronephroi	18	12	5	5
Age, days after H 24	10	17	10	14·8 ±0·48
Total length of specimen, mm.	10·17 ±0·08	14·58 ±0·20	10·10 ±0·10	13·60 ±0·24
Nose to cloaca, mm.	5·71 ±0·06	7·10 ±0·08	5·84 ±0·11	7·01 ±0·16
Ant.-post. length of pronephros	0·502 ±0·011	0·481 ±0·010	0·530 ±0·010	0·596 ±0·023
Nuclear population	1,213 ±39	1,490 ±32	1,335 ±54	1,844 ±36
Total vol. of cells, $mm.^3$	0·0077 ±0·0002	0·0081 ±0·0002	0·0095 ±0·0007	0·0121 ±0·00009
Total vol. of lumina of tubules, $mm.^3$	0·00230 ±0·00013	0·00413 ±0·00016	0·00370 ±0·00011	0·00920 ±0·00108
Overall vol. of pronephros, $mm.^3$	0·0100 ±0·0003	0·0123 ±0·0002	0·0132 ±0·0007	0·0213 ±0·0011
Area of internal surface of tubules, $mm.^2$	0·313 ±0·013	0·480 ±0·016	0·420 ±0·008	0·778 ±0·045
Vol. individual cell of pronephros, μ^3	6,394 ±168	5,478 ±113	7,113 ±309	6,579 ±87

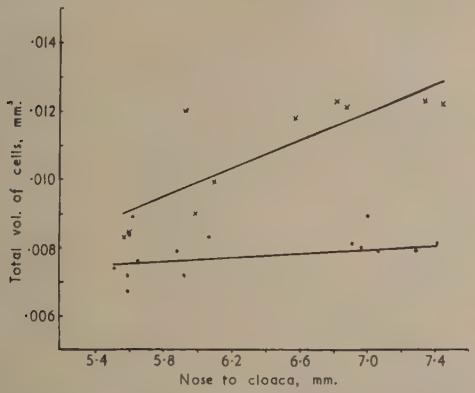
of tissue cells of 27 per cent., and the antero-posterior length of 12 per cent. The mean volume of the individual cell of a compensating pronephros shows a fall of 7·5 per cent. during the range of development studied. This, in contrast with the control groups, is not significant. In terms of nose-to-cloacal length, these changes are shown by the regression lines in Text-figs. 1–7.

Comparison between the control and unilaterally pronephrectomized groups of animals was made by an analysis of covariance to allow for the variation in tadpole length. The analysis showed that the pronephrectomized group had significantly higher values for all the measurements made (Table 2). Table 3 shows the absolute size of the means of these measurements in the control and in the pronephrectomized groups when adjusted for the size of the animal. Means are related to 6·33 mm. nose-to-cloacal length (overall mean of the two groups combined). Percentage superiority of pronephrectomized over the control larvae was as follows: means of—the nuclear population 17 per cent., total cell volume 34 per cent.; total lumen volume 100 per cent.; overall volume of pronephros

52 per cent.; internal surface area 49 per cent.; antero-posterior length 14 per cent.; and mean volume of the individual cell 15 per cent.

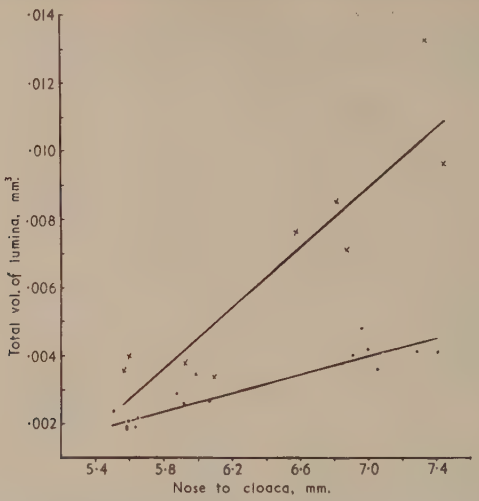


TEXT-FIG. 1. Regression lines of nuclear population of the pronephros, related to nose-to-cloacal length in mm. The dots and lower line represent the control and the crosses and the upper line represent the compensating specimens.

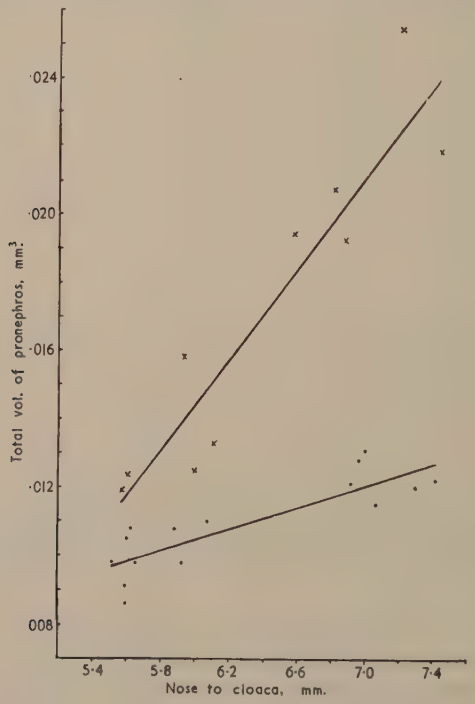


TEXT-FIG. 2. Regression lines of total volume of pronephric cells, mm.^{3}, related to nose-to-cloacal length in mm. The dots and lower line represent the control and the crosses and upper line represent the compensating specimens.

Further analysis demonstrated that there is a significant difference in the slopes of the regression lines of the two groups in all measurements, except in those relating to individual cell volume. It is apparent that in all measurements except the latter, the pronephroi of the operated animals grow faster than those of the controls.



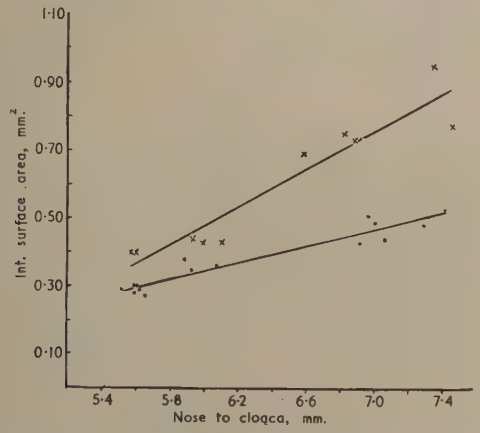
TEXT-FIG. 3. Regression lines of total volume of the tubule lumina, mm.³, related to nose-to-cloacal length in mm. The dots and lower line represent the control and the crosses and upper line represent the compensating specimens.



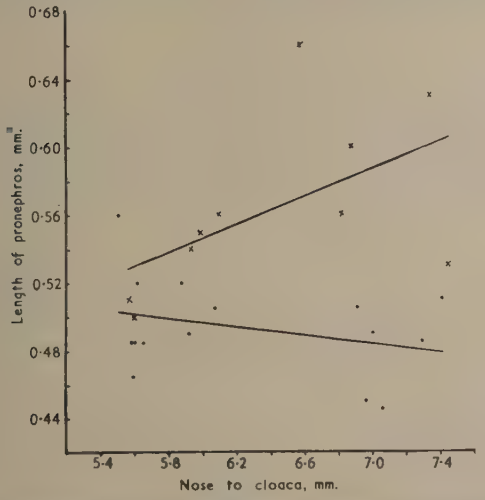
TEXT-FIG. 4. Regression lines of overall volume of the pronephros (volumes of cells and lumina together) related to nose-to-cloacal length in mm. The dots and lower line represent the control and the crosses and upper line represent the compensating specimens.

(b) The pattern of morphological change

The numerical analysis of changes which take place during control and compensating development is the most satisfactory way of demonstrating morpho-

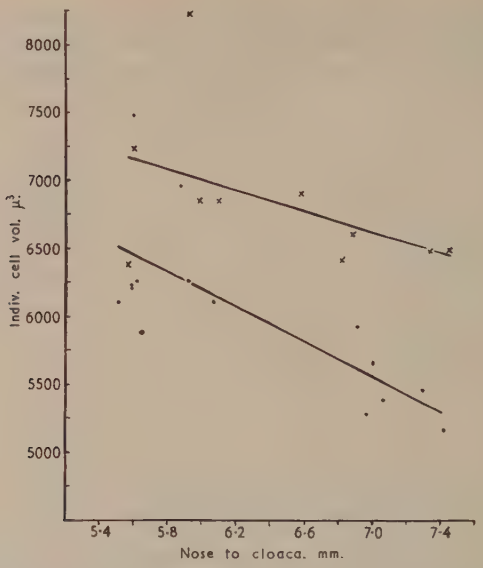


TEXT-FIG. 5. Regression lines of internal surface area of the tubules, mm.², related to nose-to-cloacal length in mm. The dots and lower line represent the control and the crosses and upper line represent the compensating specimens.



TEXT-FIG. 6. Regression lines of antero-posterior length of the pronephros, mm., related to nose-to-cloacal length in mm. The dots and lower line represent the control and the crosses and upper line represent the compensating specimens.

ogical change not always obvious from casual microscopic examination. Some eatures are particularly noticeable, however. The tubules of the unilaterally pronephrectomized group are large and inflated (Plate, figs. B, D) though the



TEXT-FIG. 7. Regression lines of individual cell volume, μ^3, related to nose-to-cloacal length in mm. The dots and lower line represent the control and the crosses and upper line represent the compensating specimens.

TABLE 2

Analysis of significance, regression, and correlation in the various measurements between control (contr.) *and unilaterally pronephrectomized* (exp.) *specimens. Thirty control and 10 experimental pronephroi*

		t *for difference between means adjusted by covariance analysis*	*Regression coefficients and standard errors*	t *for differences in regression between the two groups*	*Correlation coefficients* r
Nuclear population	contr.	4·69	±195·7±36·7	2·74	+0·8187
	exp.		+394·5±47·4		+0·9467
Total vol. of tissue	contr.	6·85	+0·0003±0·0002	2·94	+0·3503
	exp.		+0·0021±0·0005		+0·8243
Total vol. of lumina	contr.	5·20	+0·0013±0·0001	3·55	+0·9360
	exp.		+0·0044±0·0007		+0·9084
Overall vol. of pronephros	contr.	6·78	+0·0016±0·0003	3·87	+0·8555
	exp.		+0·0065±0·0008		+0·9403
Internal surf. area tubules	contr.	6·53	+0·122 ±0·010	3·57	+0·9599
	exp.		+0·277 ±0·033		+0·9466
Ant.-post. length pronephros	contr.	4·22	−0·013 ±0·010	2·22	−0·3260
	exp.		+0·041 ±0·022		+0·5465
Individual cell vol.	contr.	4·74	−640 ± 156	0·93	−0·7499
	exp.		−378±254		−0·4659

amount of swelling is variable in different specimens. Tubule walls are usually thinner than those of the controls (Plate, fig. A), although it must be remembered that the tubules in the compensating specimens not only dilate excessively, but also increase their tissue substance, which would help to counteract thinning of the tubule wall. The duct of the remaining pronephros is in practically every case

TABLE 3

Absolute size of group means when adjusted for size of animal (nose to cloaca). Overall mean nose-to-cloacal length for both groups combined, 6·33 mm.

	Controls	*Pronephrectomies*
Nuclear population.	1,340	1,562
Total vol. of pronephric cells, mm.^{3} . .	0·0080	0·0107
Total vol. of tubule lumen, mm.^{3} . .	0·0031	0·0062
Overall vol. of pronephros, mm.^{3} . .	0·0111	0·0169
Internal surface area, mm.^{2} . . .	0·391	0·582
Ant.-posterior length of pronephros, mm. .	0·493	0·563
Individual pronephric cell vol., μ^3 . .	5,994	6,900

expanded in cross-section right back to the cloaca (Plate, figs. C, E, F). This is in agreement with the findings of Howland (1916, 1921), Miura (1930 *a*, *b*), Shimasaki (1930*b*), and Cambar (1947). Simultaneously there are characteristic changes in the duct on the operated side. Of 43 operated specimens examined, 31 showed a duct atrophied in the anterior region to a mere strand of cells. This continues posteriorly as a horizontally flattened duct lying between the somite and yolk mass, and opening into the cloaca (Plate, figs. C, E, F). Twenty-two of the 31 specimens were of nose-to-cloacal length of less than 6·5 mm., and 9 were greater. Three of the younger specimens showed a tiny lumen for a short distance at the extreme anterior end. Eleven of the 43 operated specimens showed a strand-like duct completely atrophied along its length, though delicate connexions containing lumina were present joining it to the presumptive mesonephros. No lumen was present in the region where the duct joins the cloaca. Four of these 11 specimens were less and 7 greater than 6·5 mm. nose-to-cloacal length, so that they were somewhat older on the average than the previous groups. One further specimen (7·2 mm. nose-to-cloaca) had a well-developed duct with a well-formed lumen from the mesonephros backwards. This feature is probably a precocious formation of the mesonephric (Wolffian) duct developed from the pre-existing atrophied pronephric duct. We may conclude that the duct of the operated side atrophies after the extirpation of its pronephros, as found by Machemer (1929) and Van Deth (quoted by Woerdeman & Raven, 1946), and that this reduction proceeds from the anterior region posteriorly. This conclusion agrees with that of Miura (1930*a*), Maschkowzeff (1934), Shimasaki (1930*a*), and Cambar (1947, 1948), but does not confirm that of Howland (1921) who

considered that reduction began posteriorly. Unilateral extirpation of the pronephros does not affect the glomus of that side according to Howland (1921), Shimasaki (1930*b*), Van Deth (see Woerdeman & Raven, 1946), and Cambar (1949). Machemer (1929), and Maschkowzeff (1934), in contrast, considered that the glomus was smaller on the operated side. In 43 pronephrectomized specimens of *Triturus*, 17 specimens appeared to show no difference in glomus size on the two sides (12 specimens were less and 5 greater than 6·5 mm. nose to cloaca); 7 specimens had glomi practically the same size or slightly, but probably not significantly smaller (3 specimens less and 4 greater than 6·5 mm. nose to cloaca); 10 specimens showed the glomus on the operated side definitely shorter than that of the non-operated side (4 less and 6 greater than 6·5 mm. nose to cloaca); and 5 specimens showed the complete absence of a glomus on the operated side (3 less and 2 greater than 6·5 mm. nose to cloaca). Four specimens were damaged sufficiently to render the examination inconclusive. Twenty-four specimens out of 39 would thus show no size differences between the two glomi. The results would perhaps suggest that shortening or absence of the glomus after extirpation of the ipsilateral pronephric blastema is due to damage or to the removal of the presumptive glomerular tissue. If all this presumptive tissue is removed at the caudal bud stage, there is no regeneration from the surrounding tissue. Another factor to be considered is that unilateral pronephrectomy if successful often leaves a large gap adjacent to the glomus. The gut and developing lungs frequently bulge into this space and may either damage the glomus or render interpretation difficult. This does not happen on the other side. The conclusions reached would appear to be in accord with those of Howland, Shimasaki, Van Deth, and Cambar. Howland claimed that anterior and posterior nephrostomial funnels are regenerated from the coelomic endothelium in many operated embryos, and Burns (1934) and Hiller (1931) reported blind nephrostomial funnels at the site of the pronephros after extirpation of the latter and its duct rudiment in *Amblystoma*. Regeneration is denied by Machemer (1929), Hiller (1931), Maschkowzeff (1934), and Van Deth (see Woerdeman & Raven, 1946). In many operated specimens of *Triturus* (not quantitatively analysed) similar vestigial nephrostomial buds were seen. This does not necessarily mean that regeneration has taken place. There is always the likelihood that there has been incomplete removal of all the presumptive pronephric tissue, a view originally suggested by Hiller. Nephrostomial funnel regeneration is thus not proved. Extra nephrostomes were never seen in a compensating pronephros. Two nephrostomes only, as in the normal condition, were always present.

DISCUSSION

Howland (1921) measured a compensating pronephros fixed 9 days after unilateral pronephrectomy at H 30–32. One control specimen considered to be at the identical stage of development as the compensating specimen was used. She

reported increases in the following measurements: cubic content of the mass of cells 63 per cent.; nuclear hyperplasia 16 per cent.; length of tubules 21 per cent.; and the internal surface area over 100 per cent. The actual measurements of the latter were 2·037 sq. mm. (compensating pronephros) and 1·007 sq. mm. (control). Other measurements were comparative only. Hypertrophy of individual cells was also considered likely (p. 377, 1921). Within the range examined, compensatory increases in *Triturus* would seem to agree with these results in *Amblystoma*. In both genera there are small increases in nuclear population and tubule length, and substantial increases in the total volume of tissue cells and internal surface area. Individual cell hypertrophy is confirmed. Howland, however, claimed that unilateral pronephrectomy retarded growth. This is not confirmed in *Triturus*; any differences in total length or nose-to-cloacal length between control and operated specimens were not significant within the range examined.

If it is assumed that the permeability of the skin to the influx of water does not change in early larval life, then a greater volume of water will enter a larva in a unit time as development proceeds. The surplus water has to be eliminated from the larva via the pronephros. Simultaneously with increase in larval size, physiological activities associated with the pronephric tubules (e.g. absorption and secretion of metabolic products across the internal surface membranes) will increase. Increase in pronephros size would thus seem to be related to the functional needs of the larva, and there is a high degree of correlation of tubule lumina volume and internal surface area with increase in larval length.

Although no actual measurements of body volume and surface area have been made of control and compensating forms, it is extremely likely that they do not differ significantly at similar stages of development. There is no reason to believe that the permeability of the skin to the influx of water differs in these groups, and, as the larvae were preserved during life in similar osmotic media, similar quantities of water will enter larvae of the same age. As similar quantities of fluid would have to be excreted to maintain similar overall larval volumes, then it can legitimately be assumed that twice as much fluid will pass through a compensating pronephros as through one of the pair of control pronephroi. In other words, the former will do twice the work of the latter. The characteristic changes which take place during compensation must of necessity be bound up with this increased functional activity.

SUMMARY

1. Larvae of *Triturus cristatus* survive unilateral pronephrectomy at the early caudal bud stage.

2. The remaining pronephros compensates and in the main the results confirm those of Howland (1921) on *Amblystoma punctatum*.

3. A comparison between control and operated specimens by an analysis of covariance showed that the pronephros of the pronephrectomized group had significantly higher values for all the measurements made, within the range

studied (5·5–7·5 mm. nose to cloaca; 10–17 days after operation). The measurements include the means of: the nuclear population; total cell volume; total lumina volume; overall volume of pronephros; internal surface area of tubules; antero-posterior length of pronephros; volume of individual cell of pronephros. The slopes of the regression lines in the two groups demonstrate that in all measurements except that of individual cell volume, the pronephroi of the operated group grow faster than those of the controls. During development the individual cell volume significantly falls in the control pronephroi, in contrast to the operated group where the mean volume does not significantly change.

4. The duct of the compensating pronephros is expanded in cross-section along its entire length. The duct of the operated side atrophies from the anterior region towards the posterior, becoming a degenerate strand of cells, subsequently losing its lumen throughout its length.

ACKNOWLEDGEMENTS

It is a pleasure to thank Dr. H. Spurway for the supply of eggs of *Triturus*, Mr. M. Abercrombie for his critical interest and advice, Dr. K. A. Kermack and Professor P. B. Medawar, F.R.S., in whose Department the work was carried out. Photographs were prepared by Mr. W. Brackenbury, and Miss R. Birbeck assisted in preparing the manuscript. The investigation was generously supported by the Agricultural Research Council, England.

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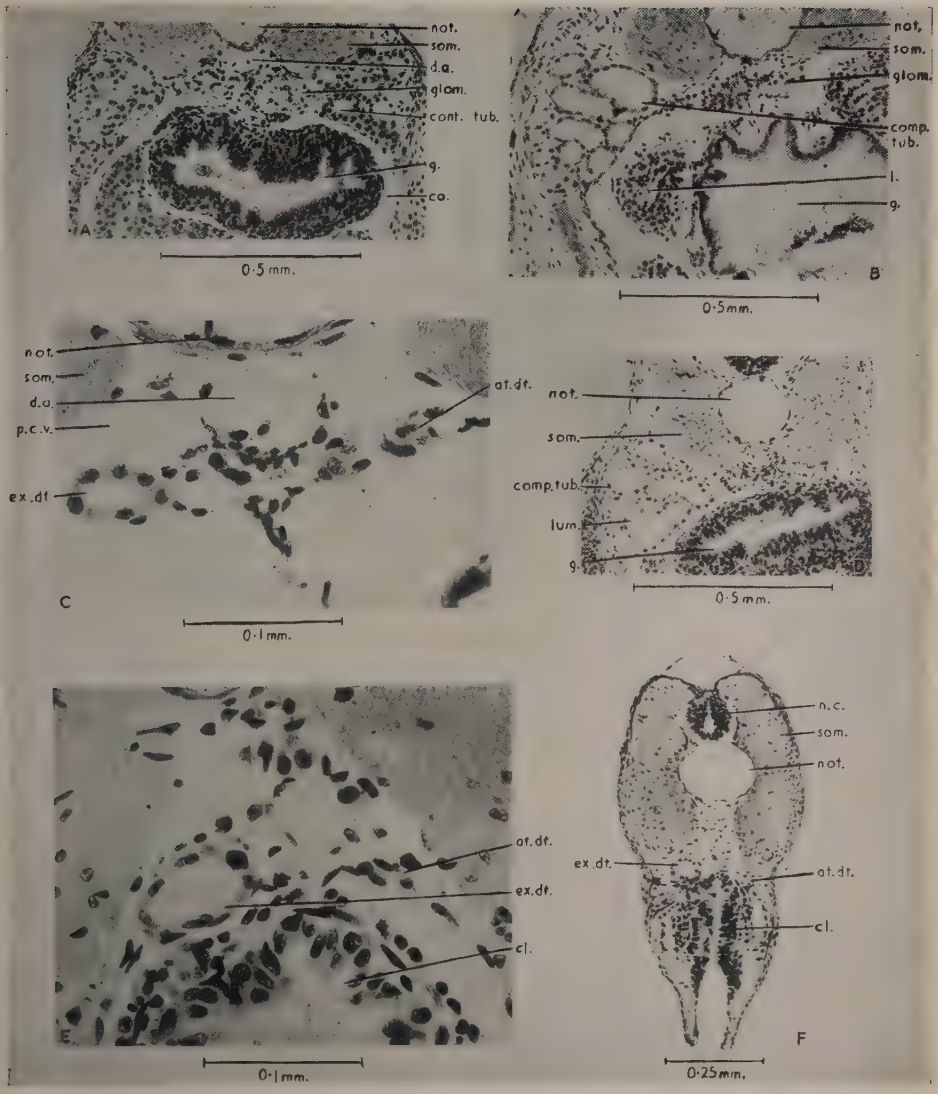
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EXPLANATION OF PLATE

Abbreviations: *at. dt.*, atrophied duct; *cl.*, cloaca; *co.*, coelom; *comp. tub.*, compensating tubule; *cont. tub.*, control tubule; *d.a.*, dorsal aorta; *ex. dt.*, expanded duct; *g.*, gut; *glom.*, glomus; *l.* lung; *lum.*, lumen of tubule; *n.c.*, nerve cord; *not.*, notochord; *p.c.v.*, posterior cardinal vein; *som.*, somite.

FIG. A. Control specimen, total length 10 mm.; nose to cloaca 5·65 mm.; length of right pronephros 0·49 mm.; and left 0·48 mm.; 10 days after wounding. Transverse section of right pronephros 0·24 mm. and left 0·18 mm. from the anterior end of the pronephros.

FIG. B. Operated specimen, total length 13 mm.; nose to cloaca 6·82 mm.; length of pronephros 0·56 mm.; 14 days after operation. Transverse section 0·23 mm. from the anterior end of the pronephros.

FIG. C. Operated specimen, total length >14·0 mm.; nose to cloaca 7·45 mm.; length of pronephros 0·53 mm.; 16 days after operation. Transverse section 3·94 mm. from anterior tip of head.

FIG. D. Operated specimen, total length 9 mm.; nose to cloaca 5·30 mm.; length of pronephros 0·54 mm.; 9 days after operation. Transverse section 0·34 mm. from the anterior end of the pronephros.

FIG. E. Operated specimen, total length 13 mm.; nose to cloaca 6·83 mm.; length of pronephros 0·55 mm.; 18 days after operation. Transverse section 6·53 mm. from anterior tip of the head.

FIG. F. Operated specimen described in Fig. D. Transverse section 5·22 mm. from the anterior tip of the head.

(Manuscript received 18:vii:55)

On Early Cell Migration Processes in the Embryonic Brain

by HARRY BERGQUIST[1]

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Head: Professor Gösta Glimstedt
and the Institute of Anatomy, University of Gothenburg
Head: Professor Bo E. Ingelmark

WITH ONE PLATE

INTRODUCTION

DURING the ontogenesis of the central nervous system, cell migration processes occur. Some of the literature on this subject has been summarized by Hamburger & Levi-Montalcini (1950) and by Bergquist & Källén (1954). When the neural tube is closed its wall is a compact neural epithelium. From it cells migrate lateralwards into a cell-free zone, formed earlier. The migration occurs from restricted areas, the migration areas, lying around proliferation furrows. The migration may take place in successive waves, forming two or more migration layers, the earlier formed lying external to the later formed. This process is called 'the process of successive migrations'. The migration layers may fuse or split secondarily, forming subdivisions that represent the rudiments of the brain nuclei. The present author (1954) has used these structures as a basis for homologizing the diencephalic nuclei, and Källén (1951) investigated the telencephalic nuclei using the same principle.

The migration processes are of different intensity in different parts of the neural tube and in different vertebrates. In Petromyzon and urodeles the migrated cells do not scatter much in the wall of the brain. In amniotes the cells spread considerably, giving rise to a relatively diffuse organization of the brain wall. In elasmobranchs and anurans a second migration layer is found inside the first one in some places. Not until reptiles is a third and even a fourth layer formed.

Herrick (1933 and later papers) showed a connexion between proliferation centres—as earlier described by Coghill (1924)—and the cytoarchitectonic organization of the brain. Bergquist (1932) found that 'die Grundgebiete [= migration areas], praktisch genommen, stets mehr oder weniger um Proliferationsfurchen herum liegen, in deren Umgebung die Zellteilung am lebhaftesten zu sein scheint' (p. 73). In a later paper (1956) it was shown that the development

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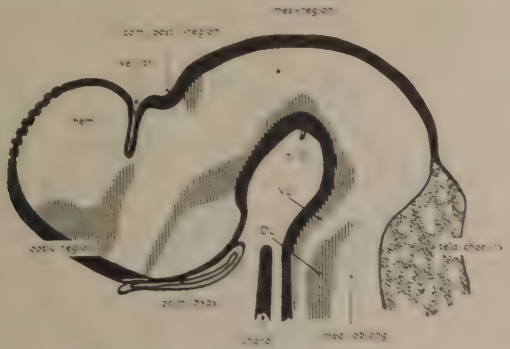
[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 152–60, June 1956]

of two migration layers in the thalamus of chick embryos is associated with two maxima of proliferative activity, each corresponding to the formation of one layer.

The object of the present paper is partly to find out where the cell migration processes start in the cerebral tube, and partly to analyse experimentally whether the process of successive migrations in the thalamus is influenced by surrounding regions of the brain.

THE EARLIEST CELL MIGRATION IN THE CEREBRAL TUBE

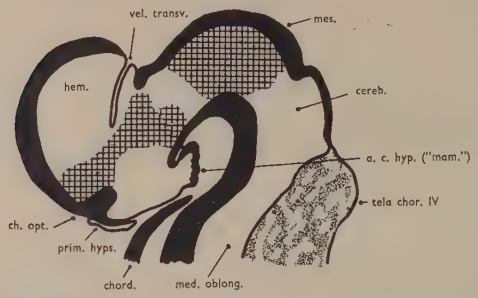
The study was made on sectioned vertebrate embryos, belonging to the collections of the Tornblad-Institute for Comparative Embryology, Lund, and the Zootomic Institute, Stockholm. Graphic reconstructions have been made. The following series have been mainly used: *Torpedo ocellata* (15–33 mm.), *Squalus acanthias* (13–26 mm.), *Cyprinus carpio* (5·4–16 mm.), *Rana temporaria* (6–24 mm.), *Lepidochelys olivacea* (7–21 days), *Gallus domesticus* (Hamburger & Hamilton's (1951) stages 17–35), *Mus musculus* (6·5–20 mm.), and *Homo* (11–34 mm.).



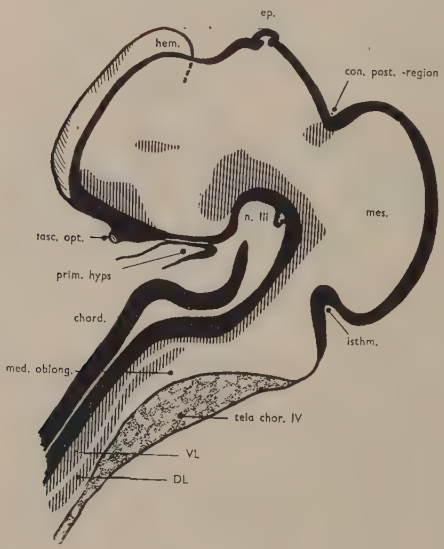
TEXT-FIG. 1. Graphic reconstruction of 17-mm. *Torpedo ocellata*, showing the brain in medial view. The hatched areas show incipient cell migration. *chord.*, chorda dorsalis; *com. post.-region*, commissura posterior-region; *DL*, dorsolateral cell column in rhombencephalon; *hem.*, hemisphere; *med. oblong.*, medulla oblongata; *mes.-region*, mesencephalon-region; *n. III*, nervus oculomotorius; *prim. hyps.*, primordial hypophysis; *tela chor. IV*, tela chorioidea ventriculi quarti; *VL*, ventrolateral cell column in rhombencephalon; *vel. tr.*, velum transversum. Magnification 25×.

Torpedo: In the 15-mm. stage a few cells migrate in the region dorsal to the plica encephali ventralis. An incipient migration can perhaps be seen in the region of the commissura posterior, the telencephalic, and the optic regions. Caudally a distinct migration exists in the ventral part of the mesencephalon and the ventrolateral cell column of the medulla oblongata.

In the 17-mm. stage the migration is quite distinct in the above-mentioned areas. It has also commenced in the dorsal hypothalamic region. A reconstruction of this stage is shown in Text-fig. 1.



TEXT-FIG. 2. Graphic reconstruction of 24-mm. *Torpedo ocellata*, showing the brain in medial view. The crossed areas show a second migration layer. *a.c. hyp. ('mam.')*, area caudalis hypothalami ('corpus mammillare' in fishes); *cereb.*, cerebellum; *ch. opt.*, chiasma fasciculorum opticorum; *chord.*, chorda dorsalis; *hem.*, hemisphere; *med. oblong.*, medulla oblongata; *mes.*, mesencephalon; *prim. hyps.*, primordial hypophysis; *tela chor. IV*, tela chorioidea ventriculi quarti; *vel. transv.*, velum transversum. Magnification 25 ×.



TEXT-FIG. 3. Graphic reconstruction of chick of stages 21–22 (Hamburger & Hamilton), showing the brain in medial view. The hatched areas show incipient cell migration. *chord.*, chorda dorsalis; *com. post.-region*, commissura posterior-region; *DL*, dorsolateral cell column in rhombencephalon; *ep.*, epiphysis; *fasc. opt.*, fasciculus opticus; *hem.*, hemisphere; *isthm.*, isthmus rhombencephali; *med. oblong.*, medulla oblongata; *mes.*, mesencephalon; *n. III*, nervus oculomotorius; *prim. hyps.*, primordial hypophysis; *tela chor. IV*, tela chorioidea ventriculi quarti; *VL*, ventrolateral cell column in rhombencephalon. Magnification 25 ×.

In the 17·5-mm. stage the cell migration has begun in still more areas, and in the 19-mm. stage most parts of the neural tube contain a migration layer. In the latter stage a new layer, the second migration layer, is developing. It is located in the dorsal thalamic, tectum opticum, and tuberculum posterior regions.

In the 24-mm. stage a second migration is visible also in the region around the optic stalk. A reconstruction is shown in Text-fig. 2.

In the 33-mm. stage a second migration can also be seen in the telencephalon. The two migration layers in the thalamus are beginning to fuse.

A similar description holds for *Squalus acanthias*.

Cyprinus: In the 7-mm. stage scattered cells belonging to the first migration wave appear in the thalamus and in the region around the tuberculum posterior. By the 7·5-mm. stage a first migration already exists in most parts of the neural tube. No second migration has been observed in this species.

Rana: In the 9-mm. stage scattered migrated cells can be observed in the thalamic and posterior tubercule regions.

Söderberg (1922) described how the striate nuclei of the telencephalon are formed in the caudal part of the hemispheres. Källén (1951) showed that they are formed as a second migration. The present author has found no other second migration.

Amniotes: The conditions in reptiles and mammals agree well with those in the chick, so only the latter will be described.

In stages 21–22 (Hamburger & Hamilton, 1951) cell migration has commenced in the preoptic region and in the ventralmost part of the neural tube: dorsal to the tuberculum posterior and in the ventrolateral cell column of the medulla oblongata. A reconstruction is shown in Text-fig. 3.

In stage 25 a second migration has commenced in the pallium of the telencephalon, while in stage 27 it is visible also in the dorsal thalamus and the synencephalic region. In stage 28 a second migration is visible in the ventrolateral cell column of the medulla oblongata, and in stage 29 also in the subpallium (striate part), and in the tectum opticum.

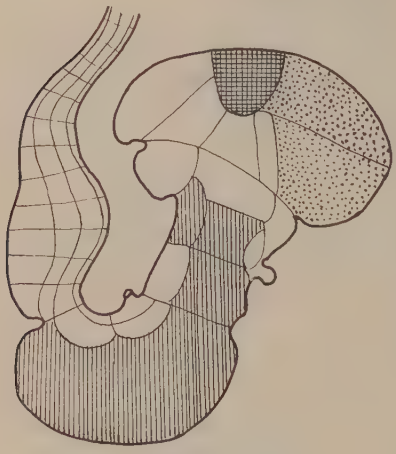
In stage 30 a third migration has commenced in the pallium of the telencephalon.

DISCUSSION OF OBSERVATIONS

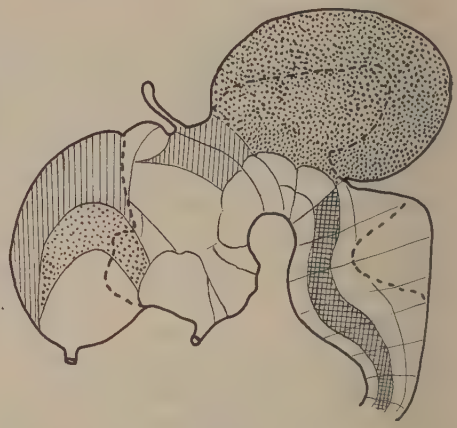
From these observations it is apparent that the first cell migration starts mainly in the ventral parts of the cerebral tube, especially around the plica encephalic ventralis. It is as yet impossible to give a convincing explanation of this fact, but some possibilities suggest themselves. At the formation of the cerebral flexure the dorsal parts of the brain expand greatly, while the volumetric increase of the ventral parts is relatively small. It would then be reasonable to assume that the newly proliferated cells in the ventral part of the tube, thus restricted, migrate lateralwards and form migration layers. But it is then surprising that in the medulla oblongata the ventral parts also show earlier cell migration than the

dorsal parts, and yet no cerebral flexure occurs in this region. Another possibility is that the proliferative rate is higher in the ventral part than in the dorsal part, and that therefore the migration can start earlier ventrally than dorsally. We have, however, no facts that indicate such a differentiated mitotic activity.

It is possible that the earlier cell migration ventrally gives a morphological substrate for an earlier differentiation of more primary functional units in the basal parts of the brain.



TEXT-FIG. 4



TEXT-FIG. 5

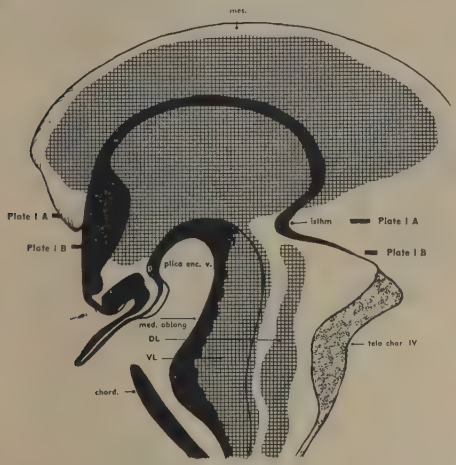
TEXT-FIG. 4. Schematic figure of the brain of a *Torpedo ocellata* embryo. The migration areas are marked. The hatched areas show the second migration at the 19-mm. stage, the crossed areas at the 24-mm. stage, and the dotted areas at the 33-mm. stage.

TEXT-FIG. 5. Schematic figure of the brain of a chick embryo. The migration areas are marked. The hatched areas show the second migration at stage 25, the crossed areas at stages 27–28, and the dotted areas at stage 29.

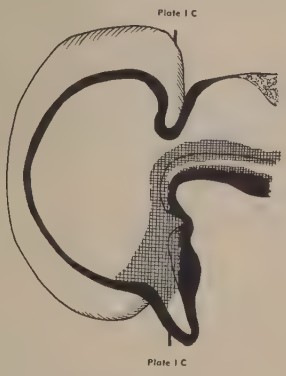
The initiation of the second migration is slightly different in *Torpedo* and in chick brains. This is schematically illustrated in Text-figs. 4 and 5. In *Torpedo* the tectum opticum, thalamus, and synencephalic regions first develop a second migration and are followed later by the telencephalon, but in the chick the pallium starts and is followed by the thalamus and synencephalon, and not until later does the tectum opticum develop a second migration. This condition is in agreement with the fact that the tectum opticum is of greater functional importance in lower animals, while the hemispheres increase in significance in higher animals. This is also in agreement with the fact that a third migration layer develops in the hemispheres and thalamus in amniotes.

These observations pose the problem whether the development of the second migration in a part of the brain, for instance the thalamus, is influenced by the surrounding regions, for instance the telencephalon and the mesencephalon.

Earlier studies by, amongst others, Hamburger (1946) and Wenger (1950) (summarized by Piatt, 1948), Hamburger & Levi-Montalcini (1950), and Hamburger (1952), have shown that the proliferation in the spinal cord is not influenced by forces intrinsic to the cord. Källén (1955) reached a similar conclusion about the regulation of the proliferation processes giving rise to proneuromery, neuromery, and postneuromery. It is, however, possible that different conditions exist for the process of successive migration. An investigation of this problem has therefore been made by the present author.



TEXT-FIG. 6



TEXT-FIG. 7

TEXT-FIG. 6. Graphic reconstruction of the brain of a chick embryo, series As, in which neuromere *a* was extirpated. The crossed areas show the second migration layers. *chord.*, chorda dorsalis; *DL*, dorsolateral cell column in rhombencephalon; *isthm.*, isthmus rhombencephali; *med. oblong.*, medulla oblongata; *mes.*, mesencephalon; *plica enc. v.*, plica encephali ventralis; *tela chor. IV*, tela chorioidea ventriculi quarti; *VL*, ventrolateral cell column in rhombencephalon. Magnification 15×.

TEXT-FIG. 7. Graphic reconstruction of the brain of a chick embryo series Ac, in which neuromere *a* was extirpated. The crossed areas show the second migration layers. Magnification 15×.

EXPERIMENTS ON THE SECOND MIGRATION IN THE THALAMUS OF CHICK EMBRYOS

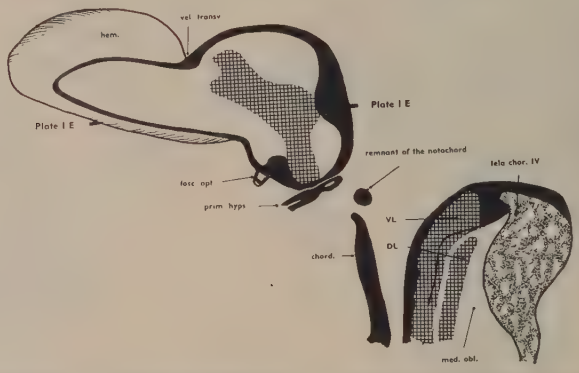
The operations were made on embryos of stages 9–15 (Hamburger & Hamilton, 1951), with from 7 to 25 somites. Two kinds of operation were performed. Either the rostralmost end of the neural tube, corresponding to neuromere *a*, was extirpated, or the mesencephalic region, neuromere *c*, was removed. For the former operation older stages were used than for the latter. The operations were made *in ovo* after vital staining, with the aid of glass needles according to the technique of Hamburger (1942). The embryos were fixed at an age of 5–6 days. Of the surviving embryos 10 were sectioned, 6 with neuromere *a* removed, and

4 with the mesencephalon extirpated. Graphic reconstructions were made of the brains and comparisons were made with normal series of corresponding age.

RESULTS

When neuromere *a* was extirpated, the thalamic region was more or less reduced in size. Reconstructions of two cases, series Ac and As, are shown in Text-figs. 6 and 7. In spite of the reduced thalamic volume, distinct second migration layers could be seen (Plate, figs. A–C). The mesencephalic wall shows a fairly normal organization with a second migration layer visible (Plate, fig. D).

When neuromere *c* (= mesencephalon) was extirpated (by Dr. Hugosson of



TEXT-FIG. 8. Graphic reconstruction of the brain of a chick embryo, series 46 M, in which neuromere *c* (=mesencephalon) was extirpated. The crossed areas show the second migration layers. *chord.*, chorda dorsalis; *DL*, dorsolateral cell column in rhombencephalon; *fasc. opt.*, fasciculus opticus; *hem.*, hemisphere; *med. obl.*, medulla oblongata; *prim. hyps.*, primordial hypophysis; *tela chor. IV*, tela chorioidea ventriculi quarti; *VL*, ventrolateral cell column in rhombencephalon; *vel. transv.*, velum transversum. Magnification 15×.

Lund), the rostral end of the neural tube developed fairly normally, and a distinct second migration could be seen in the thalamic region. One case, series 46 M, is shown as a reconstruction in Text-fig. 8, and a section through the thalamus is shown in the Plate, fig. E. In one case signs of a strong telencephalic folding, similar to the 'overgrowth' phenomenon described by Patten (1952), was observed. The present author is analysing this problem further.

CONCLUSIONS FROM THE EXPERIMENTS

These experiments seem to show that no influence is exerted on the second migration in the thalamic region either from more rostral or more caudal levels in stages later than those used for the operations. This conclusion thus agrees well with the results, mentioned above, reached by the Hamburger school. Either the second migration is caused by influences from other structures, or a determination of the process has already occurred earlier than the stage of operation.

SUMMARY

1. The earliest cell migration starts in the ventral part of the neural tube, especially around the plica encephali ventralis.

2. A survey is given of the processes of successive migrations in different parts of the embryonic brain in different species. The first appearance of the second migration layer is in different parts of the brain in *Torpedo* and chick, possibly correlated with the differing functional importance of the parts in the two species.

3. Experiments show that the formation of a second migration layer in the thalamus of chick embryos is not dependent on the presence of surrounding brain parts.

ACKNOWLEDGEMENTS

The costs of this investigation were defrayed by a grant from the Swedish Medical Research Council. The operations consisting of extirpations of neuromere *c* were made by Dr. Hugosson, Lund. I am indebted to him for lending me this material.

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EXPLANATION OF PLATE

FIG. A. Section through the thalamic region of a chick brain of series As, in which neuromere *a* was extirpated. The position of the section is shown in Text-fig. 6. The second migration layer can be seen. *migr. I*, first migration layer; *migr. II*, second migration layer; *tect. opt.*, tectum opticum. Magnification 36×.

FIG. B. Section through the foremost end of the diencephalon of a chick brain of series As, in which the neuromere *a* was extirpated. The position of the section is shown in Text-fig. 7. A second migration layer can be seen. *migr. I*, first migration layer; *migr. II*, second migration layer. Magnification 90×.

FIG. C. Section through the diencephalic region of a chick brain, of series Ac, in which the neuromere *a* was extirpated. The position of the section is shown in Text-fig. 7. A second migration layer can be seen. *mes.*, mesencephalon; *migr. I*, first migration layer; *migr. II*, second migration layer. Magnification 36×.

FIG. D. Horizontal section through the mesencephalic bulge of a chick brain, series At, in which neuromere *a* was extirpated. The second migration layer is normally developed. *a. tub. post.*, area tuberculi posterioris; *migr. I*, first migration layer; *migr. II*, second migration layer; *tect. opt.* tectum opticum. Magnification 25×.

FIG. E. Section through the diencephalic region of a chick brain, series 46 M, in which neuromere *c* (=mesencephalon) was extirpated. The position of the section is seen in Text-fig. 7. The second migration layer is well formed. *migr. I*, first migration layer; *migr. II*, second migration layer. Magnification 76×.

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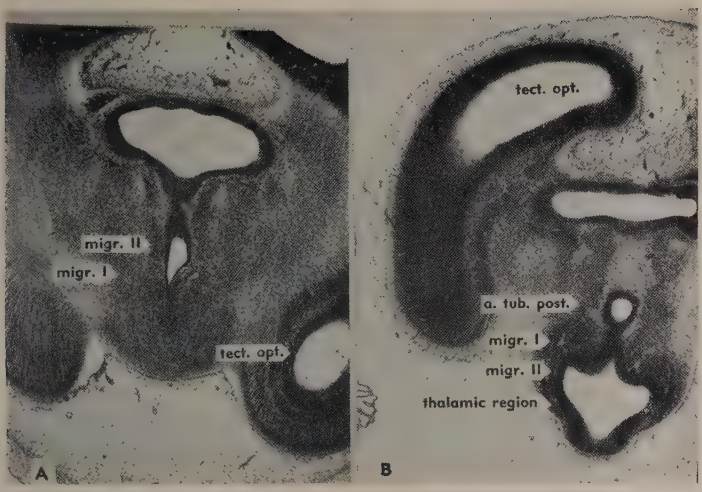
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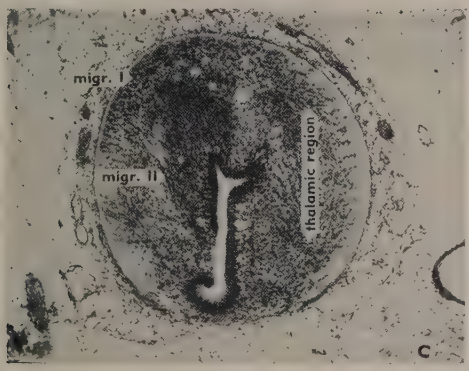
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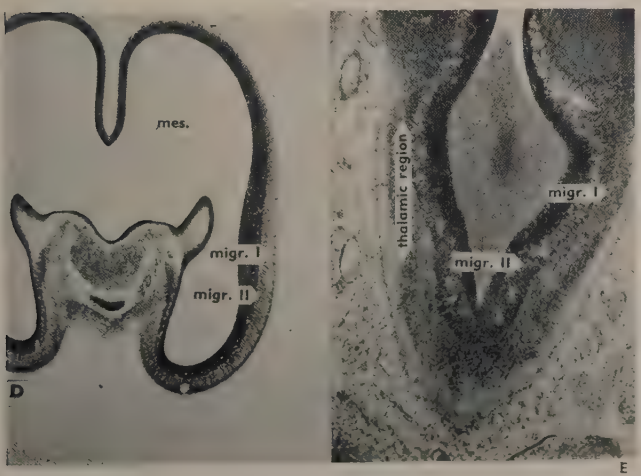
mes.
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migr. I
migr. II
E

Factors Affecting Vertebral Variation in Mice

3. Maternal Effects in Reciprocal Crosses

by ANNE MCLAREN *and* DONALD MICHIE[1]

From the Department of Zoology and Comparative Anatomy, University College London

INTRODUCTION

IT has long been realized that the biological properties and potentialities of the new-born animal are not conditioned solely by the chromosomal endowment received from its parents. Other means whereby a parental imprint may be stamped upon pre-natal development include the extra-chromosomal constituents of the gametes and, in species characterized by internal fertilization, the biological environment acting during post-zygotic existence within the mother's body.

In mammals the post-zygotic connexion between the mother and her unborn young is intimate and prolonged. Yet the number of recorded instances of 'maternal effects' in mammals is not large, while cases which have received detailed study are very few.

We are concerned to analyse maternal effects upon a skeletal character—the number of lumbar vertebrae—for which natural variation exists both in laboratory (Green, 1941) and in wild (Weber, 1950) populations of mice.

The facts which provided the basis for our investigation are the following. Whereas over 95 per cent. of mice of the C57BL inbred strain have 6 lumbar vertebrae, there are substrains of the C3H strain which have predominantly 5 lumbar vertebrae (Green, 1941). Russell & Green (1943) and Green & Russell (1951) made reciprocal crosses between mice from C3H substrains of this type and C57BL mice. They found that when the maternal parent was from the C57BL strain only 29 per cent. of the F_1 offspring had 5 lumbar vertebrae, as opposed to 57 per cent. in the reciprocal cross. The difference was manifest in the female as well as in the male offspring, and hence could not be attributed to sex-linkage. In the light of this result we resolved to investigate in detail the causes of vertebral variation within and between the two strains, with particular reference to maternal effects.

Our first step was to make an extensive survey of extant colonies of the three main substrains of the C3H strain, with results which were reported in the first

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[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 161–6, June 1956]

paper of this series (McLaren & Michie, 1954). We confirmed Green's (1953) finding that although the C3H/St and C3H/Bi substrains of the C3H strain have predominantly 5 lumbar vertebrae, the other main substrain, C3H/He, has predominantly 6. We also established that this difference has a genetic basis.

In this paper we report the results of reciprocal crosses of the C3H/Bi and C3H/He substrains both with the C57BL strain and with each other.

MATERIAL AND METHODS

Our colony of C57BL mice belongs to the C57BL/How substrain and is derived from foundation stock obtained in 1950 from Dr. Alma Howard. The foundation stock of our C3H/He colony came from the same source, but that of our C3H/Bi colony was obtained from Dr. J. Craigie. The C3H/He and C3H/Bi substrains have been separated since 1930. For a full genealogy of C3H colonies and substrains see McLaren & Michie (1954).

In all our work on vertebral type the mice have been maintained in the temperature range 65°–70° F., and fed on diet No. 86 of the Rowett Research Institute supplemented with mixed grain. Classification of vertebral type is done exclusively by X-ray photography.

TERMINOLOGY

As in the previous papers, vertebral types will be denoted by the following symbols:

5/5 = five lumbar vertebrae on each side.
6/5 = six lumbar vertebrae on the left side and five on the right.
5/6 = five lumbar vertebrae on the left side and six on the right.
6/6 = six lumbar vertebrae on each side.

When we wish to characterize a particular strain or substrain as having predominantly 5 or predominantly 6 lumbar vertebrae, we shall add the number 5 or 6 in brackets, thus—C3H/Bi(5), C3H/He(6), C57BL(6).

RESULTS

We performed all six possible types of cross between C57BL, C3H/Bi, and C3H/He mice. Data on vertebral type in the three parental populations and the six F_1 progenies are set out in Table 1.

STATISTICAL ANALYSIS

We have tested the significance of the difference between reciprocal F_1 hybrids by the χ^2 test, as shown in Tables 2, 3, and 4. There is evidence of heterogeneity between litters, since the corresponding χ^2 value is in each case greater than the number of its degrees of freedom, significantly so in the data of Tables 2 and 4. Highly significant evidence of this phenomenon has been described in other material (McLaren & Michie, 1954, 1955). We have therefore assessed the level

Table 1

Classification for vertebral type of the three parental stocks and of their F_1 offspring

Mother	Father		Vertebral types 5/5	6/5	5/6	6/6	Total	6/6 as per cent. of total
C57BL	C57BL	♀♀	0	0	0	112	112	
		♂♂	1	0	1	74	76	
			1	0	1	186	188*	98·9
C3H/Bi	C3H/Bi	♀♀	17	7	2	4	30	
		♂♂	42	2	1	0	45	
			59	9	3	4	75*	5·3
C3H/He	C3H/He	♀♀	9	2	0	58	69	
		♂♂	12	7	1	69	89	
			21	9	1	127	158†	80·4
C57BL	C3H/Bi	♀♀	6	4	3	48	61	
		♂♂	16	8	2	34	60	
			22	12	5	82	121	67·8
C3H/Bi	C57BL	♀♀	18	1	2	6	27	
		♂♂	17	5	0	2	24	
			35	6	2	8	51	15·7
C57BL	C3H/He	♀♀	2	2	1	40	45	
		♂♂	2	2	0	53	57	
			4	4	1	93	102	91·2
C3H/He	C57BL	♀♀	6	7	0	39	52	
		♂♂	10	12	2	15	39	
			16	19	2	54	91	59·3
C3H/He	C3H/Bi	♀♀	16	1	0	10	27	
		♂♂	20	3	0	3	26	
			36	4	0	13	53	24·5
C3H/Bi	C3H/He	♀♀	24	4	1	3	32	
		♂♂	21	1	0	1	23	
			45	5	1	4	55	7·3

* From McLaren & Michie (1955).
† Includes data given by McLaren & Michie (1954).

Table 2

χ^2 analysis of the results of reciprocal crosses between C57BL mice and C3H/Bi mice

	χ^2	DF	MS	
Between reciprocals	39·0129	1	39·0129	Mean square ratio = 25·472
Between litters within reciprocals . .	53·6058	35	1·5316	$P \ll 0{\cdot}001$
Total heterogeneity	92·6187	36		

of statistical significance, not directly from a table of χ^2 which might overestimate it, but from the mean squares obtained by dividing each χ^2 by the number of its degrees of freedom. The ratio of the mean squares can be assessed from a table of the variance ratio (Fisher & Yates, 1938–53, Table V).

TABLE 3

χ^2 analysis of the results of reciprocal crosses between C57BL mice and C3H/He mice

	χ^2	*DF*	*MS*	
Between reciprocals	27·8161	1	27·8161	Mean square ratio = 21·2840
Between litters within reciprocals	50·9687	39	1·3069	$P \ll 0{\cdot}001$
Total heterogeneity	78·7848	40		
Overall deviation from 77·3%*	0·1529	1		
Total	78·9377	41		

* This value was taken for the proportion of 6/6 in the two reciprocals combined. It is slightly in error owing to the inclusion of some subsequent litters. The discrepancy is of negligible effect on the statistical analysis.

TABLE 4

χ^2 analysis of the results of reciprocal crosses between C3H/Bi and C3H/He mice

	χ^2	*DF*	*MS*	
Between reciprocals	2·7787	1	2·7787	Mean square ratio = 1·5926
Between litters within reciprocals	47·1102	27	1·7448	$P > 0{\cdot}2$
Total heterogeneity	49·8889	28		

DISCUSSION

Russell and Green found a significant difference between the offspring of reciprocal crosses between C57BL(6) and C3H(5) mice. The first two F_1 items of Table 1, as shown by the analysis in Table 2, strongly confirm their finding. Table 3 shows that the difference between the offspring of the other pair of reciprocal inter-strain crosses is also statistically highly significant. Table 4 shows that there is no significant difference between the offspring of reciprocal crosses between the two C3H substrains.

Two major points of interest emerge from these data:

(1) Whereas in the crosses between C3H/Bi and C57BL the F_1 offspring are intermediate between the two parental strains, this is not so of the cross between C3H/He females and C57BL males. Expressed as the percentage showing the 6/6 type, the parental values are respectively 80·4 per cent. and 98·9 per cent. while only 64·6 per cent. of the offspring are of this type.

(2) Although the C3H/He substrain is predominantly of the 6/6 type, the females exert a maternal effect in the 5/5 direction when crossed to C57BL, just as do females of the C3H/Bi substrain which is predominantly 5/5. Judged on the crude percentage scale the C3H/He maternal effect might appear less strong than that of C3H/Bi, but on a normalized scale (McLaren & Michie, 1955) the two effects have approximately the same magnitude; the difference between reciprocals is in each case about $1\frac{1}{2}$ standard deviations.

It follows that the genetic changes which have differentiated the C3H/He and C3H/Bi substrains since their separation in 1930 have had effects on phenotype independent of those which they have had on the maternal influence. As skeletons, C3H/He mice differ from C3H/Bi mice in the 6/6 direction; as mothers, the two substrains exert maternal effects of approximately equal magnitude.

In order to determine whether the maternal effects described in this paper are exerted through the cytoplasm of the egg or through the uterine environment, we have embarked upon a programme of egg transfer from C3H donors into C57BL uterine foster-mothers.

SUMMARY

1. All six possible reciprocal crosses were made between C57BL, C3H/He, and C3H/Bi mice. The last group differs from the other two in having predominantly 5 lumbar vertebrae.

2. The offspring of the C3H/He♀♀ × C57BL♂♂ cross had a lower average number of vertebrae than either parental population. The other crosses gave offspring falling within the range between the parental means.

3. There was a significant difference between reciprocals in the two pairs of inter-strain crosses. The F_1 offspring tended towards the vertebral type of the maternal strain. No significant effect was apparent in the inter-substrain cross.

4. The evidence from the reciprocal crosses suggests that, although the C3H/He substrain has since 1930 diverged from the C3H/Bi substrain in the direction of a higher number of lumbar vertebrae, the two substrains continue to show similar maternal effects when crossed with the C57BL strain. Hence the genetic control of maternal influence upon vertebral type can operate independently of the genetic control of vertebral type itself.

ACKNOWLEDGEMENT

We wish gratefully to acknowledge the support of the Agricultural Research Council.

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(Manuscript received 2:viii:55)

Wound Contraction in Relation to Collagen Formation in Scorbutic Guinea-pigs

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INTRODUCTION

A WOUND in any mobile part of the skin of a mammal diminishes in area as it heals by a centripetal movement of the undamaged skin surrounding it. This movement, usually called wound contraction, depends on a pull exerted by the material within the wound (Lindquist, 1946; Abercrombie, Flint, & James, 1954; Billingham & Medawar, 1955). It is commonly believed that the effective force is developed by the newly formed collagen fibres. In a previous paper, however (Abercrombie, Flint, & James, 1954), we found that the course of the contraction of skin wounds in rats did not parallel the deposition of new collagen, chemically measured. This result, while certainly in no way conclusive by itself, suggested that the supposed role of collagen in contraction ought to be tested more stringently. This we have now done by measuring wounds made on guinea-pigs receiving a diet devoid of ascorbic acid. Such a deficiency largely prevents the formation of new collagen (reviewed by Wolbach & Bessey, 1942). We found, however, that it did not prevent wound contraction, a result difficult to reconcile with the usual hypothesis. We have accordingly put forward a new hypothesis as to the causal agent of contraction, which we suggest is a force produced by the population of connective tissue cells within the wound.

MATERIAL AND METHOD

In the main experiment thirty adult male guinea-pigs of mixed stock were used; for convenience of tattooing white or mainly white animals were selected. Their mean body-weight at the beginning of the experiment was 442 g. (range 378–570, standard deviation 43·4). They were tattooed under ether anaesthesia (Abercrombie, Flint, & James, 1954), eight needles being operated simultaneously to mark a square of an area, measured through the centres of the eight tattoo points, of a mean size of 25·5 mm.^{2} (range 18·1–34·4, standard deviation

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[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 167–75, June 1956]

4·11). The marks were made in the dorsilumbar region, one square on each side of the mid-line. After tattooing the animals were put on the ascorbic acid deficient diet of Murray & Kodicek (1949) with the salt mixture of Hubbell, Mendel, & Wakeman (1937), but half of them (chosen by random numbers) received in addition a supplement of crystalline ascorbic acid mixed, in the proportion of 1 g. to 100 g., into their food. After 5 days on the diet aseptic operation was performed on each animal under ether anaesthesia. A tracing of the tattoo marks was first taken on tracing paper, and a square wound of about 17 mm.^{2} was made within the tattoo points of one of the pair of squares marked on each animal, the second square remaining for estimation of changes in normal skin during the experiment. The wound extended through the panniculus carnosus to the deep fascia. No dressing was applied. Ten days later the animals were killed. The position of the tattoo marks was again marked on tracing paper, the scab removed (it was necessary to remove the larger scabs before tracing), and the new-formed tissue within the tattoo marks excised. A few animals were rejected on suspicion of infection and we were finally left with material from 14 deficient and 16 non-deficient animals.

The size of each tattoo area, marked by the centres of the tattoo points as described in our previous paper, was obtained by pricking through the tracings on to black paper, cutting out the area enclosed and measuring the transmission of light through the resulting hole photoelectrically, comparing standards of known area. The collagen content of the scabs and that of the excised tissue were obtained separately by the method of Neuman & Logan (1950), which involves estimating hydroxyproline. The results are recorded as milligrammes of hydroxyproline. They may be converted to milligrammes of collagen by multiplying by 7·46.

Mean body-weight on the first day of the diet was 429 ± 9 g. in the deficient group, 453 ± 12 g. in the non-deficient group. The difference is not significant ($t = 1{\cdot}50$, 28 degrees of freedom, $0{\cdot}2 > P > 0{\cdot}1$). Weight-loss during the 15 days of the experiment was practically the same in the two groups (the basal diet was not well taken): in the deficient group it was 46 ± 7 g., in the non-deficient group 45 ± 7 g. The initial tattoo area within which the wound was made measured $25{\cdot}3 \pm 1{\cdot}1$ mm.^{2} in the deficients, $25{\cdot}6 \pm 1{\cdot}0$ mm.^{2} in the non-deficients. In a sample of 10 of the deficient group and 8 of the non-deficient group the wet weight and hydroxyproline content of the piece excised at the initial wounding were obtained, to check that similar wounds had been made. The mean wet weights of these pieces were a little different though not significantly so (deficients $25{\cdot}4 \pm 1{\cdot}8$ mg., non-deficients $21{\cdot}3 \pm 1{\cdot}3$ mg.; $t = 1{\cdot}76$, 16 d.f., $P = 0{\cdot}1$). The excised tissue of the deficients had a mean hydroxyproline content of 633 ± 45 μg., that of the non-deficients 577 ± 33 μg., again a non-significant difference ($t = 0{\cdot}96$, 16 d.f., $0{\cdot}4 > P > 0{\cdot}3$).

RESULTS

Hydroxyproline of repair tissue

At autopsy, after careful removal of the scab, the tissue within the border of the wound marked by the tattoo was excised. It was markedly different in appearance and consistency in the deficient and non-deficient groups. In the deficient group it was very friable, and in the non-deficient control group it was quite firm. Its wet weight was similar in the two groups, with a mean of $13{\cdot}0 \pm 1{\cdot}7$ mg. in the deficient and $10{\cdot}3 \pm 1{\cdot}3$ mg. in the control group. Its hydroxyproline content was, on the contrary, highly dissimilar, with a mean of $10{\cdot}4 \pm 2{\cdot}6$ μg. in the deficient and 74 ± 8 μg. in the control group. It is clear that the vitamin deficiency was highly effective in reducing collagen formation. The small amount of hydroxyproline detected in most of the deficient animals (in 3, none was detectable) may, however, represent a trace of reticulin and collagen formed. Silver-impregnated fibres were found in a sample wound fixed in Bouin and stained with Wilder's reticulin method; but no evidence of collagen appeared with Mallory's connective tissue stain. The formation of a small quantity of argyrophilic reticulin (e.g. Bourne, 1944; Penney & Balfour, 1949) and even of mature acidophilic collagen (e.g. Hartzell & Stone, 1942; Danielli, Fell, & Kodicek, 1945) has been described in sections of wounds from scorbutic animals. Wolbach (1933) considered that such fibres do not develop in a totally vitamin C depleted guinea-pig. Since our animals were on the deficient diet for only 5 days before operation it is possible that their reserves of ascorbic acid were not entirely exhausted before healing. There is, however, the possibility to be considered that the hydroxyproline found was not present in the form of collagen but as some soluble precursor or break-down product. We tried but failed to obtain evidence in support of this possibility. In half the specimens the estimation was made of total hydroxyproline regardless of its solubility, and in half the estimation was made after extraction in 20 per cent. urea so that it represented that part of the hydroxyproline having the solubility of collagen. In the deficient group the total hydroxyproline was 13 ± 6 ($N = 8$), the collagen hydroxyproline $7{\cdot}3 \pm 2{\cdot}4$ ($N = 6$). In the control group the total was 71 ± 11 ($N = 7$), the collagen 76 ± 12 ($N = 9$). The differences within each group between total and collagen are obviously not significant. For this reason the separate types of estimation have been combined.

Scabs

There was usually a striking difference between the deficient and control animals in the scab covering the wound 10 days after operation. It was usually small both in area and thickness in the controls, averaging $3{\cdot}7 \pm 1{\cdot}4$ mg. wet weight ($N = 16$), including three where it had disappeared entirely. In the deficients it was usually very much thicker and larger in area. In this group it averaged 28 ± 6 mg. wet weight ($N = 14$), including two where it had disappeared entirely. Some of the scabs of control animals contained traces of hydroxyproline,

as found also at 10 days after wounding in rats (James, 1955): the average for those scabs still present was 10 ± 8 μg. ($N = 13$). Most of the scabs from the scorbutic animals, on the other hand, contained substantial amounts of hydroxyproline: their mean was 112 ± 23 μg. ($N = 12$). It is unfortunately impossible to determine reliably from these data whether the concentration of scab hydroxyproline was significantly higher in the deficient group or not, since only wet weights are available and water content is not likely to be comparable; and in any case the variances of the two groups are too disparate for proper comparison.

The total hydroxyproline associated with the wound, that is to say both in the scab and in the underlying repair tissue, does not differ significantly between the deficient and control groups. Their means are respectively 121 ± 23 ($N = 14$), and 82 ± 10 ($N = 16$), and comparing these $t = 1{\cdot}65$, $0{\cdot}2 > P > 0{\cdot}1$. This might raise the suspicion that in the deficient animals the hydroxyproline-containing part of the repair tissue was torn away with the large and adherent scab, which would make spurious the difference in hydroxyproline content found between the repair tissue of the deficients and controls. Some damage to the repair tissue did in fact occasionally appear to occur in this way; but the inclusion of an important amount of the hydroxyproline content of the wound with that of the scab should produce a strong negative correlation between scab and wound hydroxyproline, of which there was no trace: the correlation coefficient was positive but non-significant ($+0{\cdot}10$).

Contraction

Our previous investigation (1954) of skin wounds in rats showed that the difference between the area marked out by the centres of the tattoo points at operation and the equivalent area at autopsy gives a close approximation to (about a 20 per cent. exaggeration of) the contraction of the actual wound within the tattoo points. Measured through the tattoo points, mean contraction in the deficient group during the 10 days of healing was $8{\cdot}3 \pm 1{\cdot}0$ mm.^{2} ($N = 14$); in the non-deficient group it was $10{\cdot}1 \pm 1{\cdot}4$ mm.^{2} ($N = 16$). Significant contraction obviously occurs in the vitamin C deficient group ($t = 8{\cdot}29$, $P < 0{\cdot}001$); and the amount that occurs is not significantly different from that of the non-deficient control group ($t = 1{\cdot}02$, $0{\cdot}4 > P > 0{\cdot}3$). In three of the deficient group with *no* detectable hydroxyproline, contraction was 6·8, 9·4, and 12·5 mm.^{2} Qualitatively, the contraction appeared to be of the same nature, in that the tension was released when the content of the wound was cut free from the margin of normal skin. Our data therefore fail to provide any evidence that contraction is dependent on collagen formation. Nevertheless, the small difference actually found was in favour of the control group; and when both groups are pooled, the six wounds with most contraction all belong to the non-deficient group. It might be suspected that with larger samples the slight difference would become significant. This may be so, but the data do not suggest that such a difference could then be connected with the hydroxyproline content of the repair tissue. In fact in both deficient and

control groups wound hydroxyproline and contraction were *negatively*, though not significantly, correlated ($r = -0{\cdot}26$ in both groups). The scab might have been expected to obstruct contraction in the deficient group; and scab weight was indeed negatively correlated with contraction in this group ($r = -0{\cdot}35$) but again the relation is not significant at the 5 per cent. level of probability.

Changes in normal skin

There was a small and non-significant diminution of the area of the tattoo on the control side during the 15 days between the start of the diet and autopsy in a sample of both deficient and non-deficient groups (diminution of $0{\cdot}7 \pm 2{\cdot}2$ mm.^{2}, $N = 8$; and $0{\cdot}8 \pm 0{\cdot}7$ mm.^{2}, $N = 6$ respectively). The possibility that the deficient diet might produce detectable loss of collagen was also investigated in a small sample. No significant loss of collagen could, however, be detected when we compared the content of the piece removed at operation with the content of the control area which was removed at autopsy. At operation the mean content was 608 ± 33 μg. ($N = 9$), at autopsy 700 ± 70 μg. ($N = 9$). At autopsy the weight of the piece removed was rather higher than at initial operation, though not significantly so; probably this was the result of a slight bias in choosing the best-marked side for operation. Use of analysis of covariance to eliminate the size difference left the hydroxyproline difference still quite non-significant ($t = 0{\cdot}383$, $P = 0{\cdot}7$).

DISCUSSION

The inhibition of collagen formation in healing wounds by ascorbic acid deficiency has been demonstrated many times before (see Wolbach & Bessey, 1942), though apparently not previously by a chemical method. The absence of any effect of the deficiency on the amount of already formed collagen has been shown chemically (Elster, 1950; Robertson, 1950, 1952). The conspicuous and persistent scabs that form over wounds of scorbutic guinea-pigs were noted by Wolbach & Howe (1926), Hartzell & Stone (1942), and Danielli, Fell, & Kodicek (1945). It is not clear why they should be so different from the scabs of normal animals, but the increased fragility of the vessels (Lee & Lee, 1947) may mean a greater production of exudate; and delayed epithelialization (Danielli, Fell, & Kodicek, 1945; Galloway, Garry, & Hitchin, 1948) may allow this to accumulate. We have, however, no reason to believe that epithelialization was delayed in our specimens: the presence of the scab does not necessarily mean a failure of healing, since one specimen with a particularly large scab, which was examined histologically, had a complete and hyperkeratinized epidermis adherent to the underside of the scab. Because of the absence of collagen, such epidermis is easily torn away when the scab is removed, and the wound then looks unhealed. The presence of hydroxyproline in the scabs of wounds in rat-skin has already been demonstrated by James (1955). It remains uncertain whether scab hydroxyproline represents a diffusible potential precursor of new collagen, or comes from degeneration of some of the original collagen bordering the wound. Dévényi &

Holczinger (1954) have described the incorporation of degenerating connective tissue of the wound floor into the scab. It is not at present worth speculating on the difference in scab hydroxyproline between control and scorbutic animals. It may merely be a reflection of the curious persistence of the scab in the scorbutic animals, and not of any difference in hydroxyproline production.

The main purpose of this work was to investigate the relation between wound contraction and collagen formation. We found that wound contraction was not significantly reduced by the very severe inhibition of collagen formation that resulted from the ascorbic acid deficiency. On the average only 15 per cent. of the amount of hydroxyproline in the wounds of the controls was present in the wounds of the scorbutics, and three of the latter contracted even though devoid of detectable hydroxyproline. The contraction in both groups involved a diminution of the actual wound area by probably about 30–40 per cent., a considerable proportion, though rather less than occurs during the first 10 days in the healing of a similar-sized wound in the rat, where it is about 60 per cent. (Abercrombie, Flint, & James, 1954). The traditional hypothesis implicating newly formed collagen will not reasonably account for this contraction. Nor does there seem to be any good reason for suggesting that some intercellular component other than collagen is responsible. An intercellular substance is formed in scorbutic wounds. It has been described by Wolbach (1933), Penney & Balfour (1949), and Bradfield & Kodicek (1951). It is, however, histologically and histochemically quite different from that of normal wounds. It is appropriate therefore to suggest a new hypothesis, bearing in mind that perhaps the whole range of phenomena included under wound contraction in man and other species may require more than one explanation. The new hypothesis is that the contractile force is produced by the connective tissue cells that occupy the wound. Many authors have remarked that cellular proliferation in wounds is undiminished, or even slightly increased, by ascorbic acid deficiency (Wolbach, 1933; Hunt, 1941; Hartzell & Stone, 1942; Bourne, 1944; Meyer & Meyer, 1944). The fibroblasts may not be normal in form (Penney & Balfour, 1949) or in arrangement (Danielli, Fell, & Kodicek, 1945; Meyer & Meyer, 1944), and their immigration may be delayed (Mazoué, 1937). But, at least as far as microscopic observation extends, they are much less affected than is the intercellular substance. This is in contrast with wounds in animals treated with cortisone. Here both collagen formation and the recruitment of new cells is depressed. It is therefore not surprising, from the standpoint of the hypothesis we are putting forward, that contraction is inhibited too (Billingham, Krohn, & Medawar, 1951 *a*, *b*).

In one respect cells are a more obvious first choice for a contractile mechanism than is collagen, since neither a reticulin meshwork nor individual collagen fibres have ever been shown to be contractile under physiological conditions, but contractile mechanisms obviously occur at least in some cells. Smooth muscle-cells in tissue culture are indistinguishable from ordinary connective tissue fibroblasts; and it is conceivable that the contractile power of muscle-cells is

widespread in a primitive form throughout the fibroblast family (see Hoffmann-Berling, 1954). This line of thought, however, perhaps directs attention too much to a contractile mechanism intrinsic to the cell. Another possibility is that the force may be produced by the mutual rearrangement of cells, perhaps by expansion of the areas of adhesion which exist between cells in a fibroblast colony (Kredel, 1927), so packing them more closely together. Whatever the mechanism, there is no doubt that cells that are not muscle-cells can exert a tractive force. Mayer (1933) has shown that considerable tensions exist in fibroblast cultures which can be ascribed to the cells. Twitty (1949) has found that melanoblasts of *Triturus* under certain circumstances draw themselves together, and this is important in the formation of pigment patterns. Finally, a possibly analogous phenomenon is the contraction of blood clots, which Budtz-Olsen (1951) has shown is due not to the fibrin but to the platelets.

If it can be demonstrated that a population of fibroblasts can develop forces of the magnitude of those required to cause wound contraction, the phenomenon may have a wider significance. The embryo might in this way be provided with a source of motive power which could be a cause of the torsions, flexures, and transport of entire organs which occur in development.

SUMMARY

1. In order to investigate the relation of wound contraction to collagen formation, standard skin wounds were made on two groups of guinea-pigs, one group kept on an ascorbic acid deficient diet, the other on the same diet supplemented with ascorbic acid.

2. Ten days after wounding the amount of collagen in the repair tissue, estimated by the method of Neuman & Logan (1950), was much less in the deficient than in the non-deficient animals.

3. Nevertheless, the amount of wound contraction which had occurred, representing a loss of 30–40 per cent. of the original wound area, was not significantly different in the two groups.

4. Large amounts of hydroxyproline were found in the massive scabs commonly developed over the wounds of scorbutic animals.

5. It is suggested that the force that brings about wound contraction may be developed by the connective tissue cells of the repair tissue.

ACKNOWLEDGEMENTS

We should like to express our grateful thanks to Dr. R. P. Billingham, Dr. R. D. Harkness, Professor P. B. Medawar, and Professor J. Z. Young for their comments on the manuscript; to Mr. H. Barker and Miss Sylvia Jenkins for their technical help; and to the Nuffield Foundation for financial assistance.

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(Manuscript received 2:ix:55)

Closure of the Secondary Palate in Three Strains of Mice

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WITH FOUR PLATES

INTRODUCTION

At the time when Peter reviewed the subject of secondary mammalian palate embryology (Peter, 1924), it had been established that the secondary palate is derived from two shelves of tissue, which originate in the dorsolateral wall of the oral cavity and grow downward toward the lower jaw, passing lateral to the tongue. The shelves subsequently come to lie in the transverse plane dorsal to the tongue, where they fuse with the nasal septum and with each other, thus forming the roof of the mouth. The problem in this sequence is how the shelves manage to reach a position dorsal to the tongue after having had the tongue tightly wedged between them. Various mechanisms postulated to explain this change of position were reviewed by Lazzaro in 1940. The theories fell into three categories: (1) external forces such as muscular pressure by the tongue, (2) growth changes involving regression of the ventral portion and an outgrowth in the horizontal plane; and (3) a rapid rotation of the shelves due to some intrinsic force. Lazzaro considered the evidence to be in favour of rapid movement, and cited the cases of embryos with one shelf vertical and the other shelf horizontal as examples of a rapid transitional stage in shelf rotation. He considered his own work to indicate a swelling in the shelves due to a considerable increase of intercellular substance in the embryo's connective tissue. The result was visualized as a type of erection which would cause the shelves to rise when the obstacle of the tongue was removed. Lazzaro considered it universally recognized that the release of the tongue took place before anything else, even if the mechanism of release was 'slightly obscure'. The mechanisms suggested were: (1) a lowering of the mandible and tongue, (2) a forward displacement of the tongue, (3) a lifting of the roof of the oral cavity, (4) changes in form of the tongue due to muscular development, and (5) muscular movements of the tongue.

Thus evidence on the physiology of palate closure is scant, and even the morphology of closure is incompletely described. A further study of normal palate

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[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 176-89, June 1956]

development in mammals is desirable to provide a standard for comparison with spontaneous and experimentally induced palate abnormalities. The work presented here was carried out in conjunction with a study on cortisone-induced cleft palate, and we expect to publish the embryology of this abnormality in the near future.

MATERIALS AND METHODS

The mice used in this experiment were of strains A/Jax, C57BL/Fr, and DBA/1. They were fed Purina Fox Chow or Derwood Farms Mouse Pellets. Female mice were mated overnight when their vaginal smears indicated estrus, and were isolated the next morning if a vaginal plug was present. When calculating the age of embryos, fertilization was assumed to take place at 2 a.m. (Snell *et al.*, 1940). This system of timing is probably accurate only to within 8 hours (Lewis & Wright, 1935; Braden & Austin, 1954). Females were killed at various ages, and the embryos were placed in Bouin's fixative while still within the uterus, stored in 70 per cent. alcohol, then dissected out for gross observation or histological sectioning. The palate region can be seen readily through a dissecting microscope by removing the embryo's head and cutting away its lower jaw (see Plates 1, 2, and 3).

Live embryos were studied while clamped between two foam-rubber pads, being attached to their anaesthetized mother by their umbilical cords. The palatine shelves were observed by opening the mouth and moving the tongue with glass instruments.

RESULTS

When embryos are fixed around the time of palate closure, the embryos from one uterus will display a variety of morphological states of the palate, depending on the point at which each embryo is halted during the continuous process of palate development. These states have an obvious sequence from open palate to closed palate. For convenience, the various morphological states have been grouped into seven stages which are presented below in sequence. The stages have been artificially imposed on a continuous process, and their limits are of course entirely arbitrary. Figs. A and B in Plate 1 have been included to show the tongue in its normal position. Although the tongue is missing in the other figures, its position was observed during dissection. In the following descriptions of palatine shelf position, 'vertical' refers to the sagittal plane, and 'horizontal' refers to a plane parallel to a transverse section.

The palate shelves originate anteriorly at the point where the nasal pits join the upper part of the oral cavity and they terminate posteriorly as free knobs.

Stage 1. At the beginning of the period covered by our observations the primary palate and alveolus have already formed. The medial portion of each palatine shelf lies in the vertical plane, while laterally the shelf lies in the horizontal plane (Plate 1, figs. C and D). The tongue at first lies completely between the

shelves, but later an increase in width of the posterior part of the tongue causes it to spread out across the shelves so that an observer studying the area from a ventral position could not see the posterior ends of the shelves (Plate 1, fig. A) unless the tongue were removed (Plate 1, fig. C). Thus the tongue lies ventral to the shelves posteriorly and medial to the shelves anteriorly. At the place where it goes from ventral to medial it makes a grooved impression on the sides of the shelves. When the tongue is dissected out, the grooved impression can be seen running dorso-anteriorly on the side of each shelf (Plate 1, fig. C; Plate 2, fig. E; and right shelf in Plate 2, fig. G).

The medial sides of the shelves are not perfectly vertical, but slope somewhat. Posterior to the above-mentioned groove, the shelves slope at an obtuse angle away from the roof of the nasal cavity, so that the medial surfaces are visible when viewed from a ventral position. Anterior to the groove the shelves slope at an acute angle and thus tend to cup the tongue between them; and when the tongue is removed, the medial sides of the shelves are not fully visible to an observer looking towards the roof of the mouth (Plate 1, fig. C; Plate 2, fig. E; and right shelf in Plate 2, fig. G).

Stage 2. In Plate 2, fig. E, the groove has moved to a point midway along the shelves. This is stage 2. Plate 2, fig. F.and the right shelf in Plate 4, fig. O show cross-sections through the grooves. The medial bulges of shelf tissue are the anterior ends of the obtuse-angled slopes, and the ventral bulges are the posterior ends of the acute-angled slopes.

Stage 3. Shelf activity is not necessarily synchronized bilaterally, and a condition can be seen (Plate 1, fig. B and Plate 2, fig. G) where one shelf lies completely dorsal to the tongue while the other shelf does not. This is defined as stage 3. It should be clear from Plate 2, fig. H that the tongue has been depressed sufficiently on one side to make room for the horizontal shelf, but it seems unlikely that any actual 'rotation' of the shelf from the vertical to the horizontal position could have taken place.

Stage 4. When both shelves have assumed a horizontal position and lie dorsal to the tongue (Plate 3, figs. I, J), they are at first separated by a small space which is soon bridged by further flattening of the shelves (compare flattening left shelf with flattened right shelf in Plate 3, fig. I).

Stage 5 is the stage at which fusion of shelf epithelium begins.

Stages 6 and 7. Epithelial fusion spreads anteriorly and posteriorly (stage 6, Plate 3, figs. K, L) until the shelves are fused throughout their length (stage 7).

Preliminary to further analysis it seemed desirable to establish (1) the time in gestation at which these changes occur, (2) how long they take, and (3) whether the assigned stages do represent a chronological sequence.

The first method used to estimate the age of the embryo at palate closure was based on the calculated time of conception, and the results are shown in Table 1. In strain A/Jax, palates were open (stage 1) in all embryos examined from day 13/8 (= 13 days, 8 hours after conception) to day 14/16. However, at 14/18, nine

embryos had open palates and one embryo had a palate with one shelf in the horizontal position (stage 3). At 14/20, a litter is listed in which six embryos had closed palates, thus tending to upset the trend suggested by the other figures

TABLE 1

Relation of palate stage to chronological age

Chronological age	Strain A/Jax embryos							Strain C57BL/Fr embryos							Strain DBA/1 embryos						
	Palate stage							Palate stage							Palate stage						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
13/8	6																				
13/16	5																				
14/8	1							11	2	1	2		1		8						
/10								6			1	1	3								
/12															10						
/14								1	2		1		7								
/16	7							5			1	1			9		3	1	2	3	
/18	9		1										2	2							
/20						1	6								1					8	5
/22										1			1	7	12						
15/0	2		1	1	1	1					1			1	4						
/2	1	1				5	2							1	2	4					
/4	1		1	1		4	4														
/8	1	1	2		2																
/10																					
/12																					
/14																					
/16							2														
16/8																					
17/8																					

in the table, and raising the problem of whether or not this litter was mistimed. In general, it can be seen that, for strain A/Jax, closing stages (stages 2–6) appear in Table 1 with increasing frequency after the beginning of the 15th day. Thus the palate tends to close during late day 14 and early day 15 in strain A/Jax

embryos. Similarly, the palate appears to close in C57BL embryos from early to late day 14, and in DBA embryos during late day 14 (Table 1). The great varia-

TABLE 2

Relation of palate stage to morphological rating

Morphological rating	Strain A/Jax embryos							Strain C57BL/Fr embryos							Strain DBA/1 embryos						
	Palate stage							Palate stage							Palate stage						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
−3	11							6													
−2																					
−1																					
0								1							2						
1								1													
2	1														2						
3	1							3							13						
4								7													
5	2							3	1												
6	3							2	2		1										
7	4								2						7						
8	1									2	3				1						
9	5										1		2		2		1		1		
10	3					1					1	2	12	3	1		2		1		2
11		1	3	1								1	5					1			4
12	2	1	2	1	2	10							4	9							4
13					1		11							8							1
14														2							4
15																					
16						2															
17																					
18																					
19																					
20																					
21																					
22																					
23																					
24																					
25							1														
26																					

bility shown in this table was matched by variability in the developmental stages of embryos within the same uterus and from uteri of animals thought to have been killed at the same stage of pregnancy. (Rarely, all the embryos in one uterus were at least a day younger or older, according to Grüneberg's (1943) criteria,

than they should have been according to the calculated time of conception, in which case they were not included in Tables 1 and 2.)

Because of the irregularities in the apparent chronological age of these embryos, it was thought that a more satisfactory approach would be to estimate age by the developmental features of the embryo as described by Grüneberg (1943), modified by a further subdivision of the morphological states (e.g. webbed paws were classed as $\frac{3}{4}$ webbed, $\frac{1}{2}$ webbed, $\frac{1}{4}$ webbed). A 'morphological age' for an embryo was calculated by recording the condition of its front paws, hind paws, hair follicles, ears, and eyes, and then adding up the values that had been assigned to these conditions (Walker, 1954).

Morphological age is compared with palate stage in Table 2. The positive correlation between the progressing morphology of the embryo and the successive palate stages (Table 2) is much greater than the correlation of palate stage with assumed chronological age (Table 1). The existence of such a correlation suggests that the stages of palate closure as defined do indeed form a chronological sequence. With regard to time of closure, palate stages 2–6 start in strain A/Jax at morphological rating 10–11, whereas in strain C57BL closure starts as early as at morphological rating 5–6. In general, the palate closes sooner (by morphological age) in C57BL embryos than in A/Jax embryos, with DBA embryos being intermediate, although overlapping occurs between all three strains. Thus the strain differences suggested by chronological timing are clearer when morphological timing is used.

Having established a probable sequence of palate conditions during closure, the next step was to find the force behind this tissue movement. Mitoses can be seen in shelf tissue, but they occur too infrequently to play a significant role during the few hours (see p. 185) occupied by the closing process. Nevertheless, the head and palatine shelves were measured in fifty-two embryos; and the results were in harmony with the interpretation of tissue movement without appreciable growth. There was still the question of whether the force resided in the shelves or, for instance, in the tongue. This question was settled by the following experiments.

While examining living embryos it was noticed that the palatine shelves would often change position if the tongue was lifted. A number of genetically heterogeneous and C57BL embryos were examined on the 14th day post-conception, and it was found that the shelves often proceeded from the vertical to the horizontal position within anywhere from several seconds to a minute after the tongue was lifted. The actual movement was rapid, requiring 1 or 2 seconds; and the lack of contrast in the living embryo's mouth tissues made it very difficult to follow the movement visually. When a technique was finally evolved that produced minimal damage to the embryo and allowed clear observation of the palate region, a study was done in which observations on the living embryos were checked by fixing and re-examining these embryos. A discrepancy between the observations *in vivo* and on fixed material was found to have been caused by

reversibility of shelf movement. Sometimes, shelves that had become horizontal when the tongue was lifted, reverted to the vertical position when the mouth closed again, as would happen when the embryo was transferred to fixative.

It was found that the anterior portions of the shelves developed the ability to change shape sooner than the posterior portions. This agrees with the impression gained from morphological studies that during closure (especially stage 4) the shelves flatten out completely in the horizontal plane sooner anteriorly than posteriorly. Yet the posterior portions of the shelves are normally the first to slide dorsal to the tongue despite having less potential for movement than the anterior portions. They apparently do so because the tongue crosses the extreme posterior ends of the shelves ventrally, thus producing the arched condition that later spreads like a wave along the shelves and carries them around the tongue.

It is clear that the shelves have some potentiality for changing shape long before they would normally do so; for example, shelf movement was seen in an A/Jax embryo with a morphological rating of 5 at day 14/10, whereas palate closure is not normally expected until an A/Jax embryo has achieved a morphological rating of 10 or higher on late day 14.

Two embryos were put into 70 per cent. alcohol and examined 30 minutes later to observe the effects of alcohol on shelf tissue. Unexpectedly, it was discovered that the shelves still went from the vertical to the horizontal position after the tongue was lifted, despite having been immersed in alcohol. Furthermore, the shelves could be returned to the vertical position by pressing the mouth closed, and then caused to become horizontal again by lifting the tongue. Considering the resistance of shelf movement to alcohol, it is not surprising that there was no evidence of shelf movement having been affected by interrupted blood-supply or by mutilation of the embryo. Of course, continued treatment with alcohol eventually hardened the tissue and halted all shelf movement.

It was noticed, both with alcohol treated and untreated embryos, that the shelves could often be induced to spring back and forth between their two customary positions (horizontal and vertical), but that they would not maintain an intermediate position. This suggested a quality of elasticity; and also that two alternative stable positions existed during a certain stage of palate development, this stage falling between an early palate condition in which only the vertical position could exist and a final condition in which the shelves would force their way into a horizontal position. However, this procedure (used on embryos of appropriate age) would work only a few times with each shelf, after which the shelf would tend to remain in one of the two possible positions.

Examination of the palatine shelves in hematoxylin and eosin sections offers little to clarify the problem of what produces the force responsible for shelf movement. The palatine shelves consist of a core of loose connective tissue covered by epithelium (Plate 4, fig. N). Bone formation is occurring in the tissue proximal to the base of the shelves, and this area of bone formation parallels the shelves for a considerable distance antero-posteriorly by the 15th day. No bony

projections enter the palatine shelves until about the 17th day, and even then the bone has only entered along a short portion of the palate, and has not extended completely across it.

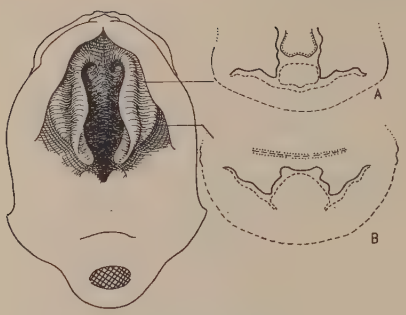
In an effort to discover substances in shelf connective tissue that could account for shelf movement, various stains were tried. Toluidine blue gave a metachromatic reaction, but instability of the metachromasia made the study of its distribution difficult. Orcein and Verhoeff's hematoxylin both stained fibres throughout the connective tissue of the head. Of the other staining procedures used (Masson's trichrome, periodic acid-Schiff, and aldehyde-fuchsin), the only one that gave a reaction with any degree of specificity for shelf tissue was Gomori's aldehyde-fuchsin stain (Gomori, 1950). The aldehyde-fuchsin appeared to be staining a network of fibres throughout the shelves (Plate 4, fig. M). Areas of cartilage and bone formation were stained, as were connective tissue fibres in some areas other than the palate; but in many regions of the head the connective tissue fibres did not stain at all with aldehyde-fuchsin (Plate 4, fig. O). Incubation in hyaluronidase (Wyeth Laboratories) for 18 hours (Bunting, 1950) rendered the shelf connective tissue unstainable with aldehyde-fuchsin, so the reacting material can be tentatively identified as an acid mucopolysaccharide.

DISCUSSION

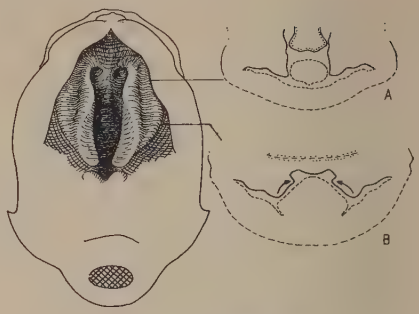
Morphology

Published information on the embryonic development of the secondary mammalian palate appears reliable except for the phase when the palate shelves change from a vertical to a horizontal position. Enough data have been assembled (Peter, 1924; Lazzaro, 1940) to indicate that the transition is too rapid to be due primarily to growth, but no satisfactory account has been offered of the mechanism by which the shelves move. In the present paper, a sequence of morphological conditions of the palate has been presented to describe how the palatine shelves can change position. Briefly, this sequence starts with the anterior portion of the tongue lying between the palatine shelves, which are hanging vertically from the roof of the mouth, and with the broad posterior portion of the tongue crossing the posterior ends of the shelves ventrally (Text-fig. 1). The change from a vertical to a horizontal position appears to start posteriorly by a bulging of the medial wall in over the tongue and an accompanying retraction of the ventral portion of the shelf (Text-fig. 2). This process proceeds in a wave-like manner anteriorly, until the whole shelf lies dorsal to the tongue (Text-fig. 3). Sometimes (perhaps always) the wave of closure passes along one shelf more rapidly than the other, thus giving rise to the condition seen by other authors (e.g. Lazzaro, 1940) of one shelf being horizontal while the other is still vertical (Text-fig. 3). When the shelves have become horizontal (Text-fig. 4) they flatten until in contact with each other (Text-fig. 5) and then fuse (Text-fig. 6).

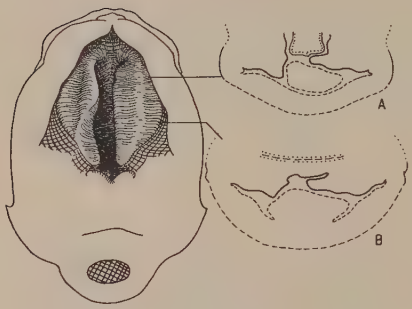
The passive role played by the tongue is emphasized by its position in histological sections of palates in which one shelf is horizontal and the other vertical



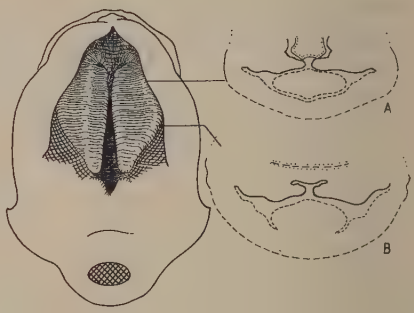
TEXT-FIG. 1



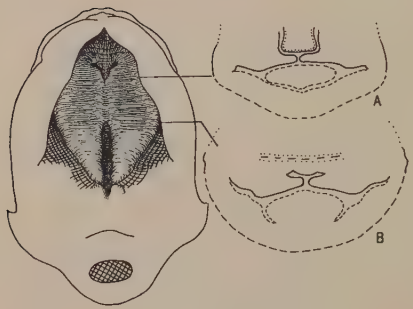
TEXT-FIG. 2



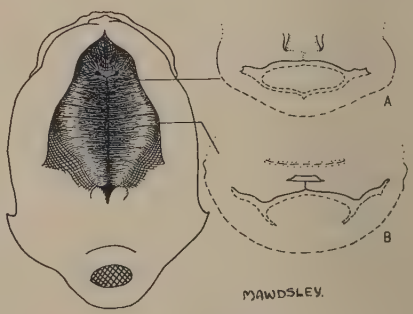
TEXT-FIG. 3



TEXT-FIG. 4



TEXT-FIG. 5



TEXT-FIG. 6

TEXT-FIGS. 1–6. In each figure the drawing on the left is a schematic representation of an embryo head with lower jaw and tongue removed; the drawings on the right (A and B) represent cross-sections taken from levels indicated by the lines connecting with the gross specimens. The arrows in Text-fig. 2B indicate the direction of tissue movement. These diagrams show the process of palate closure from a condition where the shelves are vertical (Text-fig. 1) through shelf movement (Text-figs. 2, 3, and 4) to a condition where the shelves are horizontal and fusing (Text-figs. 5 and 6).

(Plate 2, fig. H). There is no sign of the tongue having dropped to allow the shelves to become horizontal, as had been suggested by numerous authors (Peter, 1924; Lazzaro, 1940). On the contrary, the general position of the tongue remains constant, while only its shape changes, apparently in response to the change in shelf position. The side of the tongue (Plate 2, fig. H) adjacent to the vertical shelf is thick and compressed laterally, whereas the part of the tongue ventral to the horizontal shelf is more extended laterally and consequently not as thick.

Physiology

When the tongue is experimentally displaced in a living embryo of appropriate age, the shelves will move from a vertical to a horizontal position. The force within the shelves which produces this movement is presumably the same force that drives the shelves dorsal to the tongue. This shifting of shelf tissue begins posteriorly and proceeds anteriorly and is responsible for the forward movement of the grooved condition of the shelves. Finally, all shelf tissue becomes horizontal and the grooved condition disappears.

In the 20-hour period from day 14/8 to day 15/4 during which C57BL embryos were killed (Table 1), the number of closing palates seen should have been proportional to the length of time required for closing, if all palates underwent closure sometime during this period, and if the embryos were collected in equal numbers at equally spaced periods. Since these conditions were not met, the following estimation of palate closure duration is necessarily a very rough approximation of the true rate. Based on Table 1 the time required for shelf movement (stages 2, 3, and 4) is about 3 hours, and the time required for fusion (stages 5 and 6) is about 6 hours. The relatively lengthy period required for shelf movement during normal development is probably caused by the tongue's resistance to displacement, since the shelves can change position within a minute when the tongue is experimentally displaced. Experimentally induced shelf movement is reversible during a developmental stage preceding palate closure, indicating that a gradual shift of maximum stability from vertical to horizontal alignment occurs.

The idea of a rotation of the shelves (Peter, 1924; Lazzaro, 1940) is unsatisfactory, since histological study shows that the relationship of tongue to shelves does not suggest such a movement (Plate 2, fig. H), and because an intermediate shelf condition not involving rotation has been found (Plate 2, fig. F). Also, the proponents of a growth mechanism (e.g. Pons-Tortella, 1937) were incorrect regarding the force (i.e. growth and resorption) bringing about the change in shape, but they were close to being correct in their theory of how the shelves by-passed the tongue (i.e. a 'resorption' of the vertically aligned portion and an outward 'growth' of the medial wall).

Pons-Tortella (1937) reported a human embryo with palatine shelves that were in a horizontal plane anteriorly but still vertical posteriorly. He interpreted this to be a part of the palate closure process in which a transformation of the shelves

started anteriorly and proceeded posteriorly. Lazzaro (1940) discounted this finding because the embryo was damaged. Judging from Pons-Tortella's photographs, the embryo was certainly in bad condition. Yet the position of the palatine shelves was comparable to shelf position in mouse embryos whose tongues had been experimentally moved at a certain developmental stage when only the anterior portions of the shelves would stay horizontal. Thus Pons-Tortella's human embryo, which had probably received rough treatment before it could be preserved, may have had its tongue dislodged from between the shelves, allowing precocious shelf movement. The mechanism postulated by Pons-Tortella to explain shelf-transformation was resorption and growth. Although growth is presumably necessary for the building up of the force causing palate shelf movement, it certainly need not be operative during the actual movement, since the shelves have been observed in living embryos to go from a vertical to a horizontal position within one minute, with the shelves then touching each other throughout most of their length.

The metachromasia and the affinity for aldehyde-fuchsin displayed by shelf connective tissue suggests two possible mechanisms for shelf mobility. Firstly, Lazzaro's theory of tissue turgor could implicate hyaluronic acid as a water barrier, and this acid could account for the metachromasia. However, the ineffectiveness of 70 per cent. alcohol's dehydrating action on shelf movement is evidence against a turgor theory. In contrast, elastic fibres are relatively resistant to alcohol, and aldehyde-fuchsin is considered to be an elastic tissue stain (Gomori, 1950); also the presence of metachromasia is consistent with an interpretation of elastic fibres (Scott & Clayton, 1953). This suggests the second theory, namely, shelf movement is due to the tensions of a developing network of elastic fibres. The results of *in vivo* experiments on palatine shelves correlates well with a theory of an elastic fibre network which, due to changes in growth patterns, could gradually become placed under a tension. Such a system could be utilized by the embryo on other occasions. For example, the aldehyde-fuchsin staining of the maxillary and nasal processes of a day 11/10 embryo is again suggestive of an elastic fibre network (or even an elastic membrane in some areas). The fibres could serve to stiffen the processes and cause the maxillary process to bend towards the nasal process and press tightly against it in preparation for fusion.

Strain differences

A strain difference between A/Jax and C57BL embryos appears to exist in the embryonic age (measured from assumed time of conception) at which closure takes place, with C57BL palates closing about 10–12 hours before A/Jax palates (Table 1) and DBA embryos being intermediate in this respect. Because of the variability in relation of palate stage to chronological age, this difference is hard to demonstrate statistically. The less variable relation of morphological age and palate stage makes this a more suitable basis for analyzing strain differences.

When morphological ratings are tabulated with palate stages, again the trend

seen for A/Jax embryos differs from the one for C57BL. (A statistical comparison of these trends is complicated by the fact that stage 1 extends back indefinitely, and stage 7 extends onward in development indefinitely, so the only significant points in these stages are where stage 1 ends and where stage 7 begins.) The most satisfactory test of the difference would be one that utilized all the characteristics of the trends; but for convenience, a much simpler test can be used. In Table 2 it is seen that very few cases of stage 5 (shelves touching) occur, so if this stage is eliminated from calculations, two distinct groups are obtained: palates open, stages 1–4; and palates fusing or closed, stages 6 and 7. If C57BL palates usually close at an earlier developmental age than A/Jax palates, then at some particular morphological rating more palates should be closed in the one strain than in the other. It appears that by morphological rating 11, most C57BL palates have started fusing, whereas most A/Jax palates are still open. When the number of open palates and the number of closed palates up to rating 11 in A/Jax embryos (25 : 1) are compared with the same statistics in C57BL embryos (29 : 22) the two strains show a significant difference ($\chi^2 = 12{\cdot}69$, d.f. $= 1$, $p < 0{\cdot}001$). In this test only positive ratings were used, to eliminate samples from early age groups (e.g. day 13). It is true that a difference in the number of young embryos in the Stage 1 group could bias the results; but since this bias would be in favour of open palates, and since the C57BL group carried more young embryos (11 below a rating of 5) than the A/Jax group (2 below 5), it is obvious that this does not contradict the conclusion. If a similar test is performed to see whether a larger proportion of the embryos have open palates than closed palates at morphological rating 11, and later, in A/Jax (11 : 24) than in C57BL embryos (0·28), a significant difference is again shown to exist ($p = 0{\cdot}0007$ by Fisher's exact method). In this latter test, old embryos in stage 7 were slightly more frequent in A/Jax, which would have favoured a non-significant result.

CONCLUSIONS

1. The secondary palate in the mouse closes by a rapid movement of the palatine shelves from an initial sagittal to a final transverse plane. This movement consists of a bulging of the medial wall and a regression of the ventral wall of each shelf, with the transformation proceeding in a wave-like motion from the posterior ends of the shelves to the anterior ends. The flow of palatine shelf tissue into the bulge of the dorsal-medial wall carries the shelf dorsal to the tongue and forces the latter into a more ventral position.

2. The shelves move by means of an internal force, which increases until it is sufficiently strong to drive the shelves dorsal to the tongue. Evidence is presented which suggests that this force resides in a network of elastic fibres in the connective tissue of the shelves.

3. The palate closes at an earlier developmental age in C57BL embryos than in A/Jax embryos, with time of closure in DBA embryos being intermediate.

ACKNOWLEDGEMENTS

The financial assistance of the Kate E. Taylor Fund of the Banting Research Foundation, and of the National Research Council of Canada, and of the Faculty of Medicine, McGill University (Blanche Hutchison Fund) is gratefully acknowledged.

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EXPLANATION OF PLATES

Plate 1

Figs. A and B. C57BL embryo heads with lower jaws removed showing palate stages 1 and 3 respectively. Magnification × 10.

Fig. C. A/Jax embryo head with lower jaw and tongue removed showing stage 1. Magnification × 10.

Fig. D. Cross-section at the level of the nasal septum showing the palatine shelves in a vertical position. Magnification × 33.

Plate 2

Figs. E and G. A/Jax embryo heads with lower jaws and tongues removed showing palate stages 2 and 3 respectively. Magnification × 33.

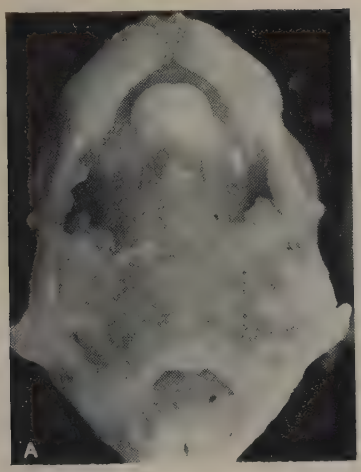
Fig. F. Cross-section at a level well back of the nasal septum (comparable to Text-fig. 2B), with lower jaw and tongue removed, showing the palatine shelves at stage 2. Magnification × 33.

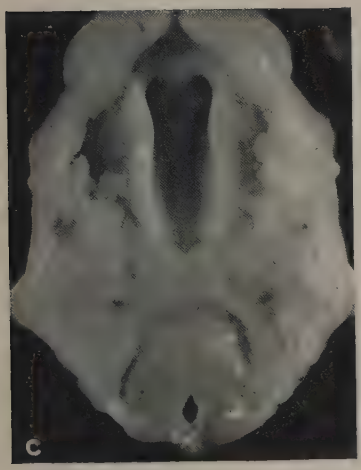
Fig. H. Cross-section at the level of the nasal septum showing the palate at stage 3. Magnification × 33.

Plate 3

Figs. I and K. A/Jax strain embryo heads with lower jaw and tongue removed showing palate stages 4 and 6. Magnification × 10.

Figs. J and L. Cross-sections at the level of the nasal septum showing palates at stages 4 and 6 respectively (tongue and lower jaw removed in Fig. L). Magnification × 33.





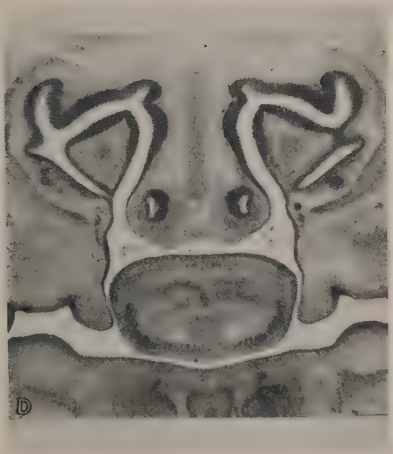


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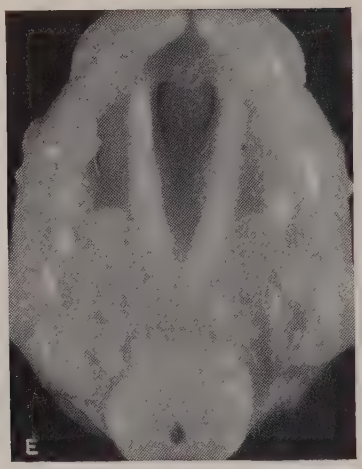
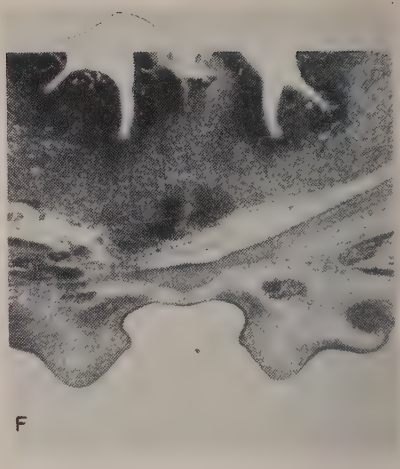
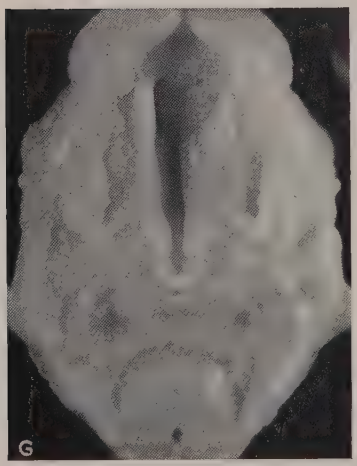
Plate 1







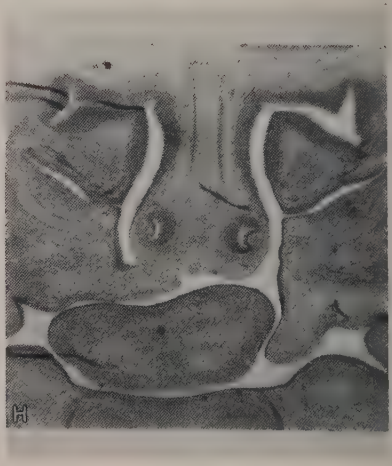


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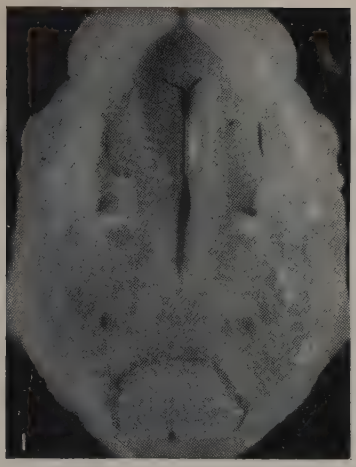
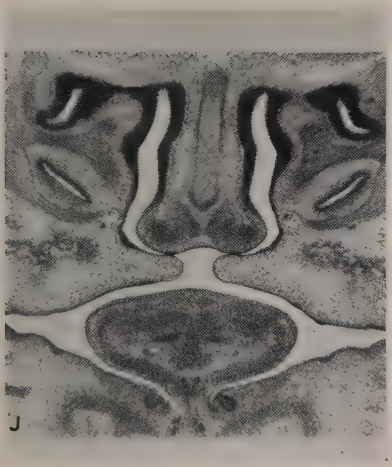
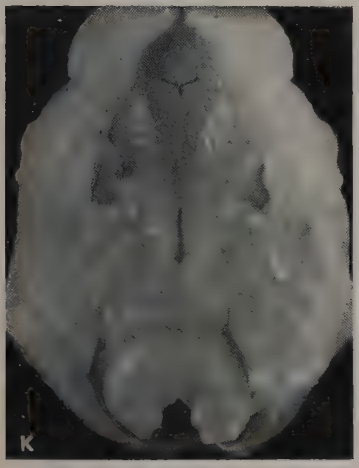
Plate 2







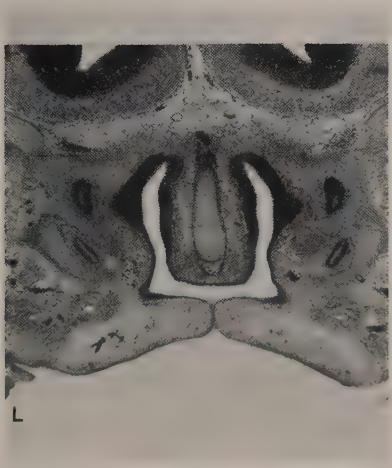


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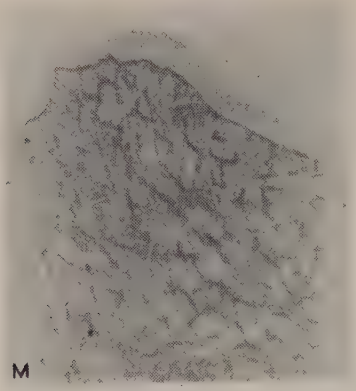
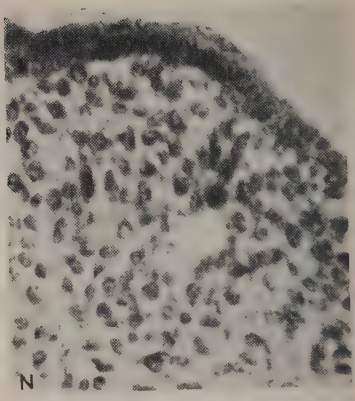
Plate 3





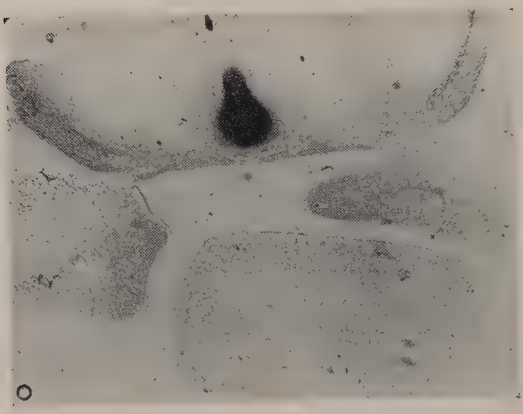


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Plate 4

PLATE 4

FIG. M. Section of palate shelf stained with Gomori's aldehyde-fuchsin. Magnification ×460. Compare with Fig. N.

FIG. N. Section of palatine shelf stained with hematoxylin and eosin. Magnification ×460.

FIG. O. Section of palatine shelf in the process of moving from the vertical to the horizontal position. Gomori's aldehyde-fuchsin stain. Magnification ×56.

(Manuscript received 20:ix:55)

A Gene for Uncomplicated Deafness in the Mouse

by M. S. DEOL[1]

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WITH TWO PLATES

DEAFNESS associated with locomotor disturbances of labyrinthine origin (head shaking, circular movements) has been known in the mouse for many years, and there are at least ten different gene mutations which produce this syndrome (with or without additional anomalies). Grüneberg (1947) remarked that

at first sight it seems remarkable that all the known types of inherited deafness in rodents should be associated with locomotor disturbances. However, shaking and circling are too obvious to be missed, and once detected such mutants will of course be tested for their sense of hearing. Deafness not so 'labelled' is unlikely to be discovered in a mouse or rat unless specifically looked for and no such systematic search has so far been made. It is thus quite likely that the absence of 'uncomplicated' deafness in rodents is spurious and simply due to the bias against their detection. The fact that there are locomotor disturbances without deafness would seem to support such a suggestion.

The gene for 'uncomplicated' deafness to be described here was first noticed through a somewhat anomalous position of the ears of these mice which is difficult to describe. A subsequent systematic search for similar conditions carried out in the mouse colony of this department resulted in the discovery of several other types of 'uncomplicated' inherited deafness which have not yet been studied in detail. It is proposed to call the condition described here 'deaf' (symbol *df*).

Hearing tests

Mice were tested for their sense of hearing at the age of 13–15 days. A sharp metallic click produced by a pair of forceps, &c., was used as a stimulus to which normal mice at once react with a flick of their ears. It is rather a rough test but it is good enough when the results can be confirmed histologically. A pinna reflex can be elicited in normal mice at the age of 13–14 days. No such reflex can be produced, as a rule, in 'deaf' (*df*/*df*) mice. There were, however, two exceptions.

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[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 190–5, June 1956]

One of these mice came from a *df*/*df* × *df*/*df* mating and must thus have been homozygous. It became deaf at the age of 2–3 months. The other animal, a male, came from a *df*/*df* × + /*df* mating and, being able to hear, was at first believed to be a + /*df* heterozygote. It was later found to have become deaf and was then tested for its genetical constitution; mated to a *df*/*df* female it produced eleven young, all deaf; it was thus very probably a *df*/*df* rather than a + /*df* mouse. Apart from the absence of the pinna reflex, the only external sign of *df*/*df* mice is a slightly abnormal posture of the ears which, although it led to the discovery of the mutant, is not easily put into words. These mice have a normal sense of equilibrium and can swim.

Genetics

The *df* gene was discovered in a strain of mice which carried a hitherto undescribed neurological condition; the presence of deafness in this stock being purely coincidental. The gene when first discovered was already widely spread in the stock and had evidently been present in it for some time.

TABLE 1

Segregation of df

	Mating	*Normal*	*Deaf*	*Total*
No. 1	*df*/*df* × *df*/*df*	—	58	58
2	*df*/*df* × +/+	70	—	70
3	+/*df* × +/*df*	194	82	276
4	*df*/*df* × +/*df*	83	119	202

Segregation data are summarized in Table 1. Matings between deaf mice produced deaf mice only (No. 1). In an outcross to unrelated normal mice (CBA/Gr) all offspring could hear normally throughout life (No. 2). The F_2 generation (No. 3) does not differ significantly from a 3 : 1 ratio though there is a slight excess of deaf animals ($P = 0{\cdot}07$). A more marked excess of deaf mice occurs in the backcross (No. 4; $\chi^2 = 6{\cdot}416$; $P = 0{\cdot}01$). This excess, if not accidental, is apparently not due to incomplete recessivity (deafness of some + /*df* mice) as all known heterozygotes had a normal sense of hearing. It might conceivably indicate an early selective advantage of *df*/*df* mice over their normal sibs.

Histology

Since the anomaly develops in post-natal life, the embryonic stages were not studied. The internal ear of deaf mice of various ages, ranging from new-born to over 450 days old, was examined histologically. Younger stages were closely spaced so as to determine the time of onset of the anomaly as exactly as possible. Normal litter-mate controls were available for most of the deaf mice examined; in other cases control mice of very nearly the same age were taken from previous experiments (Deol, 1954, 1956). The following are the different stages studied histologically ('c' denotes a normal litter-mate control):

Newborn, 3, 6, 9, 11, 14(c), 17(c), 26(c), 40(c), 70(c), 86(c), 108, 155(c), 217, 225(c), 312(c), 329 (two mice), and over 450(c) days old.

The histological technique employed has been described in a previous paper (Deol, 1954).

The anomaly in the labyrinth of *df*/*df* mice is restricted to the cochlea only; the vestibular part is quite normal. In the cochlea the following organs are affected:

Tectorial membrane

The tectorial membrane of *df*/*df* mice is normal up to the 9-day stage at which time it is nearly fully developed in normal mice (Plate 1, figs. A, B; Plate 2, fig. G). Thereafter a distortion of the membrane develops from its free end which may already be visible at the 11-day stage though sometimes the structure remains normal a little longer. This distortion increases rapidly and mice of 3 weeks or over invariably have a distorted membrane (Plate 1, fig. D; Plate 2, figs. E, F, H).

Corti's organ

The organ of Corti develops normally for the first 2 weeks. The earliest signs of degeneration can be seen at the 17-day stage when the hair-cells are considerably shortened and the nuclei of Deiter's cells have moved downwards. The shortening of the hair-cells continues until their nuclei are at the same level as the apex of the tunnel of Corti (Plate 1, fig. D). This may happen at any time from 26 to 86 days depending, perhaps, on the genetic background of the animal. Then the hair-cells disappear altogether and Deiter's cells and other supporting cells degenerate into a mass of dedifferentiated tissue (Plate 2, figs. E, F, H). The only recognizable elements left in the dedifferentiated organ of Corti are the rods of Corti. The latter remain unchanged forming a normal tunnel except in some old mice where one or both may be missing in some parts of the cochlea.

Spiral ganglion

There are no clearly visible degenerative changes in the spiral ganglion until the 26-day stage, though it is quite likely that subtle changes take place earlier. The degeneration in the beginning consists of a slight reduction in the amount of cytoplasm round the nucleus. The actual destruction of the ganglion cells does not take place until much later stages. It is always comparatively slight and far less severe than in the mutants of the shaker-waltzer group (Deol, 1954, 1956). The anomaly progresses from the basal half-turn of the cochlea upwards; though sometimes the apical half-turn is more affected than the rest. In the later stages the ganglionic mass is considerably less compact and has a clearly visible 'open' appearance (Plate 2, fig. F). The degeneration of the spiral ganglion is not progressive with age and young mice may be more strongly affected than older ones.

Stria vascularis

The degenerative changes in the stria vascularis first become visible at the 26-day stage. The cells lining the inner and outer surfaces of the stria (as seen in a longitudinal–horizontal section) are normally flattened and have fibrous processes extending inwards (Plate 1, figs. A, C). These cells tend to become rounded and to lose their fibrous processes. At the same time the amount of cytoplasm is reduced and the blood-vessels become distended or vacuoles appear. When this process is considerably advanced the stria begins to shrink. The shrinkage is usually progressive with age and may result in the stria being reduced to about one-fourth of its normal size (Plate 2, figs. I, J). But this extreme reduction is restricted, as a rule, to a part of the stria only while the rest of it is less abnormal.

DISCUSSION

The physical basis of deafness in *df*/*df* mice can be established quite convincingly except for the 14-day-old mouse. At this stage the development of the organ of Corti is complete and normal mice begin to hear. In the 14-day-old *df*/*df* mouse there was nothing clearly visible in the cochlea to which its deafness could be attributed. (At the next stage examined, which is 17 days, the organ of Corti is cleary abnormal.) As the 14-day-old *df*/*df* mouse, whose labyrinth was sectioned, was deaf, this suggests that functional defect of the cochlea precedes visible changes in it. That was also found to be the case in the double heterozygotes of the shaker-1 and waltzer genes (Deol, 1956). On the other hand, in both cases the possibility remains that subtle structural defects might have been revealed by different histological techniques. To account for the exceptional *df*/*df* mice which can hear for a period it must be assumed that normal structure of the cochlea can persist for some time in these mice.

The pathology of the cochlea in the mutant *df* is remarkably similar to that of the cochlea of the mutants waltzer, shaker-1, shaker-2, jerker, Varitint-waddler, and pirouette (Deol, 1954, 1956). There are, of course, differences in the degree of degeneration and the time of onset of the anomaly, just as the same kind of differences exist between the various mutants in the shaker-waltzer group. To what extent these differences are differences between the genes themselves or between the genetic backgrounds in which they find themselves could only be decided by comparing the various genes on an isogenic background. But the cost in time and labour of such an undertaking would be prohibitive.

No tests have so far been carried out as to whether the *df* gene is an allele of one of the genes of the shaker-waltzer group. Similarly, it remains to be studied whether double heterozygotes of *df* and genes of the shaker-waltzer group interact in a similar way as the genes waltzer and shaker-1, where the double heterozygotes become deaf in middle life (Lord & Gates, 1929; Deol, 1956). It has been suggested by Grüneberg, Hallpike, & Ledoux (1940) that this interaction is due to the similarity of the pathological processes in the cochlea in these mutants. If

so, there are good reasons to expect that the *df* gene might react with some of the other genes of that group.

It has been observed that in some fish and amphibians the macula of the sacculus takes part in the acoustic function of the labyrinth (Löwenstein, 1936). As the sacculus of *df*/*df* mice is structurally normal it cannot have any acoustic function in the mouse.

SUMMARY

1. A new recessive gene has been discovered in the mouse which produces deafness without any locomotor disturbances.

2. *df*/*df* mice are usually deaf from the beginning. However, there were two mice which could hear for some time but became deaf later.

3. The deafness in *df*/*df* mice is caused by a degeneration of the organ of Corti, the spiral ganglion, and the stria vascularis.

4. The pathology of the cochlea closely resembles that of the shaker-waltzer mutants.

ACKNOWLEDGEMENTS

I am grateful to Dr. H. Grüneberg, F.R.S., for his constant encouragement and criticism. My thanks are due to my wife for technical assistance. The work was carried out under grants from the Agricultural Research Council and the Rockefeller Foundation which are gratefully acknowledged.

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EXPLANATION OF PLATES

All the figures are from longitudinal–horizontal sections of the cochlea. The magnification given is approximate.

Plate 1

Fig. A. Cochlear duct; normal; 9 days old; ×155.

Fig. B. Cochlear duct; deaf; 9 days old; ×155.

Fig. C. Cochlear duct; normal; over 450 days old; ×155.

Fig. D. Cochlear duct; deaf; 70 days old; ×155.

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Plate 1

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Plate 2

PLATE 2

FIG. E. Cochlear duct; deaf; 155 days old; ×155.
FIG. F. Cochlear duct; deaf; 329 days old; ×155.
FIG. G. Corti's organ; deaf; 9 days old; ×225.
FIG. H. Corti's organ; deaf; 155 days old; ×225.
FIG. I. Stria vascularis; normal; over 450 days old; ×325.
FIG. J. Stria vascularis; deaf; over 450 days old; ×325.

(Manuscript received 26:ix:55)

Recherches expérimentales sur la nature et les propriétés de l'inducteur de la régénération des yeux de la planaire *Polycelis nigra*

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[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 196–216, June 1956]

INTRODUCTION

Des recherches expérimentales antérieures (Lender, 1952) avaient montré que la régénération des yeux de la planaire *Polycelis nigra* était induite par le cerveau. En effet, après la destruction de cet organe et l'excision des yeux latéraux, la régénération des ocelles ne se produisait pas. Mais l'activité inductrice du cerveau s'exerçait à distance sans intervention des liaisons nerveuses et sans qu'il y eût contact physique entre l'organe inducteur et les néoblastes qui se différenciaient en cellules visuelles (Wolff, 1953). L'ensemble des expériences suggérait l'hypothèse que l'activité inductrice du cerveau serait de nature hormonale et s'exercerait par l'intermédiaire de substances chimiques: les organisines. Ces substances de neurosécrétion, décelées dans la région antérieure du corps de la planaire, manifestaient les propriétés suivantes: elles ne possèdent pas de spécificité zoologique et elles sont sécrétées par le cerveau normal ou en voie de nécrose (Lender, 1951).

Pour vérifier l'hypothèse des organisines de la régénération des yeux, j'ai entrepris de nouvelles expériences dans le laboratoire d'embryologie expérimentale de la Faculté des Sciences de Strasbourg sous la direction de Monsieur le Professeur Étienne Wolff.

Il fallait tout d'abord établir avec certitude l'existence des organisines dans le corps de la planaire. Les techniques utilisées pour obtenir les résultats précédents (greffes de bord oculé, irradiations aux rayons X) devenaient insuffisantes: la technique des broyats m'a permis de mettre en évidence quelques propriétés de la substance inductrice.

En premier lieu j'ai essayé de déterminer l'influence des conditions expérimentales sur l'activité de l'organisine: sa pénétration dans le corps de la planaire et l'influence de sa concentration sur la régénération des yeux.

Je me suis aussi posé la question de la nature chimique de l'organisine. Sans arriver à répondre d'une manière définitive à cette question, j'ai pu déterminer un certain nombre de propriétés qui pourront servir de point de départ pour de nouvelles recherches.

Enfin j'ai expérimenté avec des broyats de queues de planaires. Les expériences de greffes de bord oculé dans la région caudale avaient montré que le pouvoir inducteur du cerveau ne s'y faisait pas sentir. J'ai essayé de vérifier ce résultat tout en cherchant s'il n'était pas possible d'activer cette région non inductrice. Des recherches analogues avaient été faites sur l'organisateur des Batraciens. Dans le développement embryonnaire des Amphibiens, de nombreux auteurs avaient montré que des tissus non inducteurs, comme l'épiderme présomptif par exemple, pouvaient être activés par un traitement approprié (par exemple l'action de la chaleur ou de l'alcool; Bautzmann, 1932; Holtfreter, 1932; Spemann, 1932).

MÉTHODES

L'étude de la régénération des yeux a été faite sur *Polycelis nigra* (Ehrbg.). Cette planaire possède de nombreux yeux marginaux. La régénération des yeux latéraux excisés débute vers le 7ème jour, à la température de 18°, sous l'influence inductrice du cerveau. L'excision de cet organe empêche la différenciation des ocelles. Le mode opératoire est le suivant: la planaire anesthésiée au chlorétone à 0,2 pour cent est placée sur une couche de paraffine sous le binoculaire. On

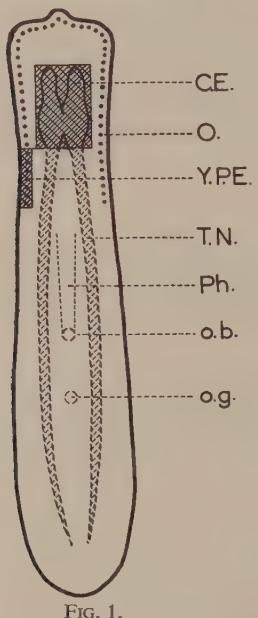

FIG. 1.

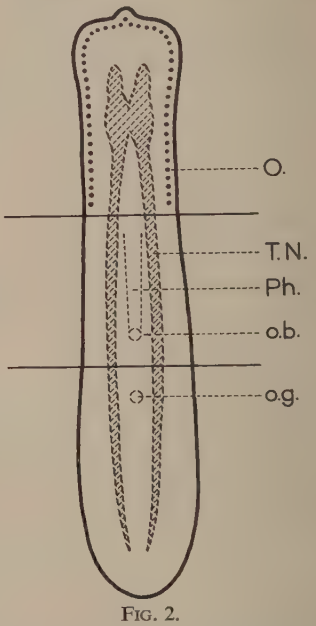

FIG. 2.

FIG. 1. Schéma de l'expérience d'excision du cerveau et des yeux latéraux de *Polycelis nigra*. *C.E.*, cerveau excisé; *O.*, œil; *Y.P.E.*, yeux latéraux excisés; *T.N.*, tronc nerveux; *Ph.*, pharynx; *o.b.*, bouche; *o.g.*, orifice génital.

FIG. 2. Les différentes régions du corps de *Polycelis nigra* utilisées pour la confection des broyats: la tête avec les yeux, *O*; la région pharyngienne avec le pharynx, *Ph.* et la bouche, *o.b.*; la région caudale avec l'orifice génital, *o.g.*; *T.N.*, tronc nerveux latéral.

excise le cerveau et les dix derniers yeux latéraux de gauche (Fig. 1). Le troisième, le cinquième et éventuellement le septième jour, le blastème de régénération du cerveau est de nouveau excisé. Les planaires opérées sont élevées dans des godets: 4 planaires dans 10 c.c. d'eau. L'eau d'élevage est renouvelée au moment de l'excision du blastème de régénération du cerveau. Les produits, dont on veut étudier l'influence sur la régénération des yeux, sont ajoutés à l'eau d'élevage. Grâce à cette méthode j'ai pu contrôler le pouvoir inducteur de différents types de broyats de planaires.

Un premier type de broyats est obtenu en hachant finement au scalpel une partie du corps de la planaire. On obtient des broyats de tête en prélevant la région antérieure jusqu'à la hauteur des ovaires, des broyats de la région pharyngienne en prélevant la région médiane du corps; et des broyats de la région caudale en prélevant la partie du corps située en arrière de l'orifice génital (Fig. 2).

Des expériences ont été faites avec des broyats de *Polycelis nigra*, *Dugesia lugubris*, *D. gonocephala*, et *Dendrocoelum lacteum*.

Le broyat normal est fait à partir de 10 à 15 tronçons ainsi définis. Il est mis en suspension dans 10 c.c. d'eau contenant 4 *Polycelis* en expérience. Des broyats de 5 ou de 20 parties de planaires ont aussi été étudiés. Dans certains cas ces broyats ont été traités par la chaleur. Le broyat en suspension dans l'eau est chauffé à 60°, 80°, ou 100° pendant 2 ou 30 minutes. D'autres broyats ont été traités par l'alcool à 70° ou 95°. Le broyat est mis dans quelques c.c. d'alcool. Puis le liquide est évaporé à l'air et le dépôt est mis en suspension dans l'eau d'élevage des *Polycelis nigra*.

Les constituants solubles dans l'eau peuvent être isolés grâce à la méthode des broyats au sable. Quinze parties de planaires sont broyées pendant quelques minutes dans un mortier en présence de sable pour analyse et 10 c.c. d'eau. Puis le broyat est conservé à la glacière à 2° pendant 5 heures. Ensuite on le centrifuge à 10 000 tours/minute pendant 20 minutes. Le liquide surnageant limpide est recueilli et utilisé comme milieu d'élevage des *Polycelis* en expérience.

Les *Polycelis nigra* témoins sont opérés comme les autres planaires (excision du cerveau et des yeux latéraux de gauche) mais on les élève dans l'eau ordinaire sans addition de broyat.

Tous les individus ayant régénéré des yeux ont été fixés au Zenker. Les coupes de 5 μ d'épaisseur ont été colorées au Mallory. On contrôle ainsi la présence ou l'absence du cerveau chez les planaires opérées.

L'ACTIVITÉ INDUCTRICE DES BROYATS DE TÊTES

(A) *Mise en évidence de l'organisine dans les broyats de têtes de* Polycelis

Si la régénération des yeux de *Polycelis nigra* se fait par voie hormonale, sous l'influence d'une organisine, l'inducteur doit conserver son activité, même si les cellules du tissu sont tuées. Les expériences d'irradiation aux rayons X de la région antérieure de *P. nigra* avaient déjà montré que la régénération des yeux était possible même si la plupart des cellules nerveuses étaient en dégénérescence.

On peut obtenir un tissu fortement lésé en fabriquant un broyat haché: tout d'abord j'ai élevé des *P. nigra* en présence de broyats de têtes hachées. Les résultats expérimentaux sont résumés dans le Tableau 1.

La régénération des yeux de *P. nigra* en présence des broyats de têtes hachées est donc normale puisqu'un seul individu sur 29 n'était pas oculé le 9ème jour de l'expérience. Sur les 28 individus oculés, une planaire avait régénéré un cerveau

long de 100 μ, capable d'induire la régénération des yeux ainsi qu'il résulte de mes travaux antérieurs (Lender, 1952). Six individus possédaient du tissu nerveux sur une longueur de 20 à 50 μ. Mais des expériences antérieures avaient montré que cette masse cérébrale était trop petite pour provoquer la régénération des yeux. Donc dans 27 cas l'apparition des ocelles est due à la présence des broyats de têtes. Ceux-ci contiennent l'organisine de la régénération des yeux. Cette substance doit diffuser dans l'eau d'élevage, car normalement les broyats ne servent pas de nourriture aux planaires.

TABLEAU 1

Régénération des yeux sous l'influence d'un broyat de têtes hachées

Nombre de planaires en expérience		*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
Au début	*A la fin*		*Nbre de planaires*	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
36	29	1 planaire	28	1 à 3	7–9	1 6	100 20–25–30–50–50–50	27 planaires

Nous avons donc la preuve que l'intégrité du tissu inducteur (le cerveau) n'est pas nécessaire à la régénération des yeux, qui est induite à distance par l'intermédiaire de l'organisine.

(B) *La Diffusion de l'organisine des broyats de têtes*

Si l'organisine diffuse dans l'eau il doit être possible de séparer la substance active des tissus broyés.

(1) *Broyats enrobés dans la gélose*

Les *Polycelis nigra* opérées sont élevées dans l'eau sur une couche de gélose contenant un broyat de têtes hachées. La couche de gélose et l'eau d'élevage sont

TABLEAU 2

Régénération des yeux en présence de broyats enrobés dans la gélose

Type d'expérience	*Planaires en expérience*	*Pas de régénération d'yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
			Nbre de planaires	*Nbre d'yeux*	*Délai de régénér.*	*Nbre de planaires*	*Longueur (en μ)*	
Broyats enrobés dans la gélose	12	2	10	1–3	7–9 j.	2	20–25	10
Sur fond de gélose	10	7	3	1–2	7–9 j.	3	85–100–125	—
Sans gélose	12	10	2	2–3	7–9 j.	2	90–135	—

renouvelées tous les deux jours au moment de l'excision du blastème de régénération du cerveau. Une partie des planaires témoins est élevée sur une couche de

gélose sans broyat incorporé, l'autre partie est élevée en l'absence de gélose. Les résultats numériques sont résumés dans le Tableau 2.

L'élevage des *P. nigra* sur fond de gélose pure n'influence pas la régénération des yeux. C'est le cerveau régénéré qui est la cause de l'apparition des ocelles chez les 3 planaires. Au contraire l'incorporation d'un broyat de têtes à la gélose a provoqué la régénération des yeux chez la plupart des individus. Les quantités de tissu nerveux régénéré sont très minimes (20 et 25 μ). L'organisine doit donc diffuser dans l'eau à travers une couche de gélose.

(2) *Broyats au sable centrifugés*

On peut encore extraire l'organisine des broyats de la manière suivante. Un broyat au sable est centrifugé. Le liquide surnageant sert de milieu d'élevage pour les planaires en expérience. Les résultats obtenus sont résumés dans le Tableau 3.

TABLEAU 3

Régénération des yeux sous l'influence de l'organisine obtenue par centrifugation d'un broyat de têtes

Type d'expérience	*Planaires en expérience*	*Pas de régénération d'yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
			Nbre de planaires	*Nbre d'yeux*	*Délai de régénération (jours)*	*Nbre de planaires*	*Longueur (en μ)*	
Avec surnageant	24	3	21	1 à 3	7–9	2 2	80–100 30–35	19
Témoins	24	18	6	1 à 2	7–9	6	90–95–100–110–115–150	—

Si on compare les Tableaux 1 et 3 on constate que le surnageant de la centrifugation est aussi actif que le broyat haché. L'organisine a passé en totalité dans l'eau. L'induction de la régénération des yeux se produit en l'absence de toute cellule provenant de l'organisateur. L'organisine est donc une substance chimique. Elle est soluble dans l'eau ou tout au moins diffuse très facilement en milieu aqueux.

(C) *Conditions de l'activité de l'organisine des broyats de tête*

Dans les expériences précédentes, les planaires étaient en contact avec l'organisine pendant toute la durée de la régénération des yeux. Comment se déroule alors la régénération des yeux si l'organisine n'est présente que pendant un laps de temps plus court, ou si on fait varier la concentration de la substance inductrice?

(1) *Étude de l'activité inductrice des broyats en fonction du temps de contact*

(*a*) *Les planaires sont en présence des broyats pendant un jour sur deux.* Les planaires dont on veut étudier la régénération sont en contact avec les broyats un jour sur deux. Dans une expérience les broyats de têtes hachées sont ajoutés

à l'eau d'élevage au moment de l'excision du cerveau ou de son blastème de régénération, c'est-à-dire les 1er, 3ème, et 5ème jours de l'expérience. On les laisse en contact pendant 24 heures. Dans une autre expérience les broyats ne sont ajoutés que 24 heures après l'excision du cerveau ou du blastème de régénération, c'est-à-dire les 2ème, 4ème, et 6ème jours. Les résultats numériques, résumés dans le Tableau 4, montrent que dans les deux cas la régénération des yeux est normale. Les chiffres sont voisins de ceux consignés dans le Tableau 1.

TABLEAU 4

Régénération des yeux de Polycelis. *Les broyats sont actifs pendant un jour sur deux*

Période d'action des broyats	*Planaires en expérience*	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
			Nbre de planaires	*Nbre d'yeux*	*Délai de régénération*	*Nbre de planaires*	*Longueur (en μ)*	
1er, 3ème et 5ème j.	24	2	22	1 à 3	7–9 j.	6	60–60–80–95–125–150	16
2ème, 4ème, et 6ème j.	24	3	21	1 à 3	7–9 j.	3	100–100–100	18
Témoins	36	30	6	1 à 3	7–9 j.	6	90–100–100–90–90–100	—

Dans ces expériences on peut constater que la proportion de planaires ayant régénéré du tissu nerveux est plus importante. L'influence de l'organisine sur la régénération des yeux n'a donc pu être mise en évidence avec certitude que chez un nombre un peu plus faible de planaires (16 sur 22 et 18 sur 21).

Ces résultats font aussi ressortir la grande perméabilité des planaires à l'organisine. Si on ajoute le broyat au moment de l'excision du cerveau ou de son blastème de régénération, l'organisine doit sûrement pénétrer dans la planaire par la blessure. Si on ajoute les broyats le lendemain de l'excision, l'organisine ne peut pas pénétrer par la blessure, déjà cicatrisée dans la majorité des cas. Elle doit arriver dans les tissus de l'animal, soit par la bouche, soit par la région cicatrisée de la blessure.

(*b*) *Les planaires sont en contact avec les broyats pendant 4 ou 2 jours.* La présence de l'organisine n'est pas nécessaire pendant toute la durée de la régénération des yeux. On peut alors chercher à déterminer le moment où sa présence est indispensable.

Les *Polycelis nigra* sont élevées en présence de broyats de têtes hachées. Dans un premier type d'expériences les planaires sont en contact avec les broyats pendant les 4 jours qui suivent l'excision des yeux. Le broyat est ajouté le 1er jour, renouvelé le 3ème jour et éliminé le 5ème jour. Dans un autre type d'expériences les planaires sont en contact avec les broyats pendant 2 jours seulement, soit le 1er et le 2ème jour, soit le 3ème et le 4ème jour après l'excision des yeux. Les résultats numériques sont résumés dans le Tableau 5.

On constate que la régénération des yeux est normale si l'organisine est pré-

sente pendant les quatre premiers jours de l'expérience. Mais le nombre de planaires, chez lesquelles on constate la régénération des yeux, est plus faible si l'organisine n'est présente que pendant deux jours. Dans ce cas, les meilleurs résultats sont obtenus si on fait agir les broyats entre le 3ème et le 4ème jour.

TABLEAU 5

Régénération des yeux de Polycelis. *Les planaires sont en contact avec les broyats pendant 4 ou 2 jours*

Période d'activité du broyat	*Planaires en expérience*	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence du broyat*
			Nbre de planaires	*Nbre d'yeux*	*Délai de régénération (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
1er au 5ème j.	23	4	19	1 à 3	7–9	1	100	18
1er et 2ème j.	17	11	6	1 à 2	7–9	2	60–60	4
3ème et 4ème j.	24	9	15	1 à 4	7–9	2	100 et 200	13
Témoins	43	32	11	1 à 3	7–9	11	70–70–80–80–90–90–90–100–100–100–120	—

Donc l'organisine exerce son activité inductrice sur les néoblastes entre le 1er et le 4ème jour de la régénération des yeux, avec un maximum d'activité vers le 3ème et le 4ème jour. Sans doute à ce moment la majorité des cellules de régénération est en place dans le bord oculé et est capable de réagir à l'action inductrice.

Nous comprenons aussi pourquoi le délai de régénération des yeux est le même pour les planaires dont le cerveau est laissé en place et pour les planaires dont on a excisé le cerveau en même temps que les yeux. Dans ce dernier cas le cerveau se régénère vers le 3ème ou 4ème jour et peut alors induire la différenciation des ocelles.

(2) *Influence de la concentration de l'organisine*

On peut tenter de définir la concentration d'organisine efficace en fonction du nombre de têtes broyées en suspension dans 10 c.c. d'eau. Dans les expériences précédentes l'activité inductrice de l'organisine a été contrôlée en élevant 4 planaires en présence d'un broyat de 10 à 15 têtes dans 10 c.c. d'eau. La concentration de l'organisine n'a donc pas varié. Comment se déroule la régénération des yeux si on fait des élevages en présence de broyats plus concentrés ou plus dilués?

Dans ces expériences on utilise le surnageant de la centrifugation de broyats au sable. Les broyats sont faits à partir de 5 têtes ou de 20 têtes. Les résultats numériques sont indiqués dans le Tableau 6.

Ce tableau fait ressortir nettement que la concentration des broyats, c'est-à-dire la concentration de l'organisine dans l'eau d'élevage, influence la régénération des yeux de *Polycelis nigra*.

TABLEAU 6

Influence de la concentration de l'organisine sur la régénération des yeux de Polycelis

Nombre de têtes broyées dans 10 cc. H^2O	*Planaires en expérience*	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
			Nbre de planaires	*Nbre d'yeux*	*Délai de régénération (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
5 . .	12	6	6	1–2	7	3	75–75–110	3
20 .	24	1	23	1–2	5–7	1?	50	22
0 broyat (témoins)	36	27	9	1–2	7	9	75 à 200	—

N.B. pour 10 à 15 broyats voir le Tableau 3.

Comme le montre le Tableau 3 on se rapproche des conditions normales de la régénération en extrayant l'organisine de broyats faits à partir de 10 à 15 têtes pour un élevage de 4 planaires dans 10 c.c. d'eau. Presque tous les individus régénèrent alors de 1 à 3 yeux entre le 7ème et le 9ème jour. Si on diminue la concentration de l'organisine en n'utilisant que des broyats de 5 têtes, un quart seulement des planaires régénère des yeux dans le même délai. Si au contraire la concentration de l'organisine est plus forte, la régénération des yeux débute plus tôt. Avec des broyats de 20 têtes, elle est en avance de 48 heures chez 50 pour cent des individus; et le 7ème jour la régénération des yeux a débuté chez tous les individus. Mais nous constatons aussi que seule la vitesse de régénération est influencée et non le nombre d'yeux régénérés puisque le début de la régénération des yeux est toujours marqué par l'apparition de 1 ou 2 ocelles. Les travaux sur la régénération chez les planaires avaient montré que la différenciation des ocelles était précédée par une phase de migration des néoblastes vers le bord excisé; en présence d'une concentration élevée d'organisine la différenciation des yeux se fait sans doute plus rapidement. Mais on ne peut exclure l'hypothèse que l'organisine pourrait aussi favoriser la migration des néoblastes.

(D) *Influence de l'alcool et de la chaleur sur l'organisine*

Nous savons qu'une lésion des cellules ne supprime pas le pouvoir inducteur dans un tissu contenant normalement l'organisine (Lender, 1951). En tuant toutes les cellules de l'organisateur, par l'alcool ou la chaleur par exemple, on peut contrôler s'il y a persistance du pouvoir inducteur. On voit aussi comment se comporte l'organisine vis-à-vis de ces agents.

(1) *Action de l'alcool*

L'action de l'alcool à 70° a été étudiée sur des broyats de 10 à 14 têtes hachées. Le Tableau 7 fait apparaître que le pouvoir inducteur subsiste après traitement des broyats par l'alcool. Le pourcentage d'individus qui régénèrent les yeux est plus faible qu'avec un broyat non traité (voir le Tableau 1). On peut penser que

TABLEAU 7

Régénération des yeux sous l'influence de broyats traités par l'alcool

Planaires en expérience	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
		Nbre de planaires	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
20	6	14	1–2	7–9	2	70–100	12
Témoins 16	13	3	1	7–9	3	90–100–100	—

l'alcool exerce sur l'organisine une faible action dégradante. Mais en tout cas après l'action de l'alcool et la dessiccation l'activité de l'organisine est encore très grande. Ce résultat est analogue à celui qui fut obtenu par Spemann, Fischer, & Wehmeier (1933) dans le traitement de l'inducteur du développement des Batraciens.

(2) *Action de la chaleur*

L'influence de la chaleur sur l'organisine a été étudiée sur des broyats de 10 à 14 têtes hachées, chauffés pendant 2 minutes à 60°, 80°, et 100° ou pendant 30 minutes à 100°.

TABLEAU 8

Influence de la chaleur sur l'organisine

Traitement du broyat	*Individus en expérience*		*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
	Début	*Fin*		*Nbre de planaires*	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
Chauffage à 60°	28	27	3	24	1 à 3	7–10	1 2	100 30–35	23
Chauffage à 80°	12	12	3	9	1 à 2	7–9	1 1	120 15	8
2 minutes ébullition	24	23	11	12	1 à 3	7–8	5	80–100–100–125–80	7
30 minutes ébullition	12	12	11	1	1	7–8	1	85	0
Pas de broyat (témoins)	68	68	52	16	1 à 3	7–8	16	90 à 140	—

Le Tableau 8 montre que l'activité inductrice des broyats diminue si on soumet les tissus à des températures de plus en plus élevées. Le pouvoir inducteur des broyats chauffés à 60° est presque intact (comparaison avec le Tableau 1).

Au fur et à mesure qu'on soumet les broyats à des températures plus élevées (80° et 100°) le pouvoir inducteur devient de plus en plus faible sans disparaître complètement. Le tiers des planaires régénère encore les yeux après un chauffage à 100° pendant 2 minutes. Si l'on veut rendre l'organisine inactive, il faut faire bouillir les broyats dans l'eau pendant 30 minutes environ.

La substance inductrice présente donc une très nette thermolabilité aux températures supérieures à 80°.

(E) *La non spécificité zoologique de l'organisine*

Des expériences de greffes avaient montré que l'action inductrice du cerveau n'était pas spécifique. Le cerveau du *Dugesia lugubris* pouvait induire la régénération des yeux dans un greffon de bord oculé de *Polycelis nigra*. Mais ces expériences sont délicates à réaliser; le tissu greffé ne reste que rarement en contact avec l'hôte; la migration des néoblastes entre le porte-greffe et le greffon n'a pas lieu si on s'adresse à 2 espèces différentes de planaires. J'ai repris ces expériences en élevant les *Polycelis* en présence de broyats de têtes hachées de *Dugesia lugubris*, *D. gonocephala*, et *Dendrocoelum lacteum*.

TABLEAU 9

Régénération des yeux de Polycelis *sous l'influence de broyats hétérogènes*

Origine des broyats	*Planaires en expérience*	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
			Nbre de planaires	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
Dugesia lugubris	24	5	19	1–3	7–9	4	80–90–100–100	15
Dugesia gonocephala	30	9	21	1–3	7–9	3	70–70–100	18
Dendrocoelum lacteum	16	11	5	1–2	7–9	3	100–130–150	2
Témoins	68	52	16	1–4	7–9	16	100 à 150	—

Le Tableau 9 montre que la régénération des yeux se produit chez un nombre plus ou moins grand de *Polycelis* sous l'influence des broyats hétérogènes. Les taux de régénération sont plus faibles que dans les expériences avec des broyats de *Polycelis*. Les meilleurs résultats ont été obtenus avec les broyats de *Dugesia lugubris*. Les broyats de *Dendrocoelum lacteum* n'ont qu'une activité inductrice très réduite. Mais ces expériences confirment les résultats antérieurs. Les tissus d'autres espèces de planaires contiennent aussi de l'organisine qui est donc une substance chimique dépourvue de spécificité zoologique.

L'ACTIVITÉ INDUCTRICE DES BROYATS DE RÉGIONS PHARYNGIENNES ET CAUDALES

Les expériences de greffes de bord oculé avaient montré que l'activité inductrice du cerveau n'était décelable que dans la région antérieure du corps. Un greffon de bord oculé transplanté près du cerveau régénérait les yeux. Sur un

greffon transplanté dans la région pharyngienne ou la région caudale les ocelles ne se différenciaient pas. On pouvait donc admettre que l'organisine n'était présente que dans la région antérieure du corps. Les expériences avec les broyats ont confirmé l'activité inductrice de la partie antérieure du corps. Mais la méthode des broyats permet aussi de contrôler le degré d'activité inductrice des régions pharyngiennes et caudales.

(A) *Broyats de régions pharyngiennes*

Les broyats sont faits à partir de 15 régions pharyngiennes hachées.

Le Tableau 10 montre qu'en présence de broyats de régions pharyngiennes hachées, la régénération des yeux est très faible. Mais les planaires gardent leur aptitude à régénérer les yeux. Si le blastème de régénération du cerveau n'est plus excisé les ocelles se différencient dans un délai de 7 à 8 jours.

TABLEAU 10

Activité inductrice de la région pharyngienne

Planaires en expérience		*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
Début	*Fin*		*Nbre de planaires*	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
20	17	15	2	1–2	7–9	0	—	2

(B) *Broyats de régions caudales*

(1) *Broyats inactifs de régions caudales*

Les broyats sont faits à partir de 15 régions caudales hachées de *Polycelis nigra* ou *Dugesia gonocephala*.

Le Tableau 11 montre que les broyats de régions caudales hachées sont peu actifs. Dans 1/10 des cas seulement, les yeux se sont différenciés sous l'influence

TABLEAU 11

Activité inductrice de broyats de régions caudales hachées

Type de broyat	*Individus en expérience*		*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
	Début	*Fin*		*Nbre de planaires*	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
Polycelis . .	32	29	25	4	1–2	7–9	1	100	3
Dugesia gonocephala	12	12	11	1	2	8	—	—	1

de substances inductrices contenues dans les broyats. L'organisine semble exister dans tout le corps de la planaire. Mais sa concentration décroîtrait si on s'éloigne de la région cérébrale. Nous serions alors en présence d'un gradient de concentration de l'organisine.

(2) *Broyats activés de régions caudales*

Les expériences sur le développement de l'œuf d'amphibien ont montré que des tissus normalement dépourvus du pouvoir organisateur pouvaient être activés par des traitements appropriés. C'est pour cela que j'ai essayé d'éveiller le pouvoir inducteur dans les broyats de queues en les traitant par la chaleur, l'alcool ou en écrasant les tissus avec du sable.

(*a*) *Broyats traités par la chaleur.* J'ai étudié la régénération de *Polycelis nigra*, élevées en présence de broyats de queues hachées chauffés à 60° pendant 2 minutes. En même temps j'ai élevé des planaires en présence de broyats de queues non chauffés et des planaires en l'absence de broyats. Le Tableau 12 résume les résultats numériques.

TABLEAU 12

Régénération des yeux de Polycelis *en présence de broyats de queues chauffés à 60°*

Type de broyat	Planaires en expérience		Pas de régénération des yeux	Régénération des yeux			Régénération du cerveau		Régénération des yeux sous l'influence des broyats
	Début	Fin		Nbre de planaires	Nbre d'yeux	Délai (en jours)	Nbre de planaires	Longueur (en μ)	
Chauffé à 60°	60	52	16	36	1–3	7–9	3	100–125–150	33
Non chauffé	48	45	37	8	1–2	7–9	5	70–70–80–100–150	3
Pas de broyat (témoins)	20	20	12	8	1–3	7–9	8	100 à 200	—

Nous constatons que les broyats de queues hachées chauffés à 60° sont beaucoup plus actifs que les broyats non traités par la chaleur. Plus de la moitié des planaires régénère les yeux en présence de broyats chauffés. La chaleur a donc éveillé le pouvoir inducteur dans la région caudale. Cependant l'efficacité des broyats de régions caudales chauffés à 60° est plus faible que celle des broyats de têtes traités de la même manière (Tableau 8, première ligne).

Que devient le pouvoir inducteur si on chauffe plus fortement ou plus longtemps les broyats de queues? Les broyats de queues hachées sont chauffés pendant 2 minutes à 80° ou 100° et pendant 30 minutes à 100° de la même manière que les broyats de têtes.

Le Tableau 13 montre que le pouvoir inducteur des broyats de queues diminue régulièrement après chauffage de 60° à 100°. Pour obtenir une disparition presque totale de l'organisine, il faut chauffer les broyats vers 100° pendant 30 minutes. L'organisine des broyats de queues se comporte à la chaleur comme l'organisine des broyats de têtes (voir Tableau 8). Il semble donc que dans les deux cas nous soyons en présence de la même substance.

Pourquoi l'activité inductrice apparaît-elle dans les broyats de queues après l'action de la chaleur?

Deux hypothèses se présentent à l'esprit: la queue de la planaire contiendrait

une substance inactive qui, sous l'influence de la chaleur, se transformerait en une organisine capable de provoquer la différenciation des ocelles. Mais on pourrait aussi admettre que l'organisine existe normalement dans les cellules de la queue sous forme de réserve immobilisée. La chaleur jouerait alors le rôle d'un agent capable de provoquer la libération de la substance. L'une ou l'autre hypothèse pourrait fournir l'explication des résultats précédents.

TABLEAU 13

Régénération des yeux en présence de broyats de queues chauffés

Traitement du broyat	*Planaires en expérience*		*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
	Début	*Fin*		*Nbre de planaires*	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de Planaires*	*Longueur (en μ)*	
Chauffé à 80°	12	12	3	9	1–2	7–9	3	60–70–80	6
2 minutes à 100°	12	11	6	5	1–2	7–9	0	—	5
30 minutes à 100°	24	23	15	8	1–3	7–9	6	75–100–135–150–160–165	2
Pas de broyat (témoins)	24	24	17	7	1–3	7–9	7	75 à 140	—

(*b*) *Broyats traités par l'alcool.* Dans les broyats de queues peut-on mettre en évidence l'organisine après traitement par d'autres agents que la chaleur, par exemple l'alcool?

Les broyats, obtenus en hachant les queues de planaires, sont traités par l'alcool à 70° ou 95°. Les planaires, dont on veut vérifier la régénération, sont élevées en présence de ces broyats.

TABLEAU 14

Régénération des yeux sous l'influence d'un broyat de queues traité à l'alcool

Planaires en expérience	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
		Nbre de planaires	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
18	9	9	1–3	7	1	100	8
Témoins 12	10	2	1	7	2	100–125	—

Le Tableau 14 montre que, sous l'influence de l'alcool, les broyats de queues manifestent aussi leur pouvoir inducteur bien que le nombre d'individus régénérant les yeux soit plus faible qu'en présence des broyats de queues chauffés à 60°. L'alcool exerce donc sur ces broyats la même influence que la chaleur.

Il est peu vraisemblable qu'une substance inactive devienne active sous l'influence d'agents aussi différents que la chaleur et l'alcool. Il est plus simple d'admettre que les deux traitements provoquent la libération d'une substance déjà présente. Les expériences avec des broyats de queues centrifugés confirment cette hypothèse.

(*c*) *Broyats au sable centrifugés.* Les broyats au sable de 15 queues sont centrifugés comme les broyats de têtes. Les planaires en expérience sont élevées en présence du liquide surnageant.

Le Tableau 15 montre que le surnageant de queues broyées au sable possède aussi une activité inductrice notable. Cette activité est comparable à celle d'un broyat de queues traité par la chaleur à 60° (voir le Tableau 12).

TABLEAU 15

Régénération des yeux sous l'influence du surnageant de queues broyées au sable

Planaires en expérience	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
		Nbre de planaires	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
24	10	14	1–2	7–9	2	50–60	13
Témoins 24	17	7	1–3	7–9	7	80 à 200	—

On peut donc éveiller l'activité inductrice des yeux dans les broyats de queues de *Polycelis nigra* en faisant agir soit la chaleur modérée, soit l'alcool, soit le broyage au sable. Mais qu'y a-t-il de commun entre ces trois types d'expériences? Dans les 3 cas les cellules sont détruites. La perméabilité sélective de la membrane cellulaire disparaît et les substances contenues dans la cellule peuvent diffuser librement à l'extérieur. Il semble que la substance inductrice existe dans la queue, soit sous la forme masquée, soit sous la forme active. Normalement l'organisine ne diffuserait pas dans la région caudale. La substance organisatrice, présente dans la région caudale, se comporte dans les expériences comme l'organisine extraite de la tête de la planaire. Il semble donc, dans les deux cas, qu'on soit en présence d'une même substance.

On peut admettre que dans la planaire *Polycelis nigra* en régénération, l'organisine émise par le cerveau diffuse librement dans la région antérieure, c'est-à-dire dans la zone oculée, mais reste bloquée dans les cellules du reste du corps. Les broyats de 15 queues traités par la chaleur ou l'alcool sont moins actifs que des broyats de 15 têtes. On peut penser que la concentration de l'organisine dans les broyats de queues doit être plus faible que dans les broyats de têtes. L'étude de l'influence de la concentration des broyats de queues sur la régénération des yeux va le confirmer.

(3) *Influence de la concentration des broyats de queues*

On broie au sable les régions caudales de *Polycelis* et on centrifuge le broyat. Les planaires dont on étudie la régénération sont élevées dans le liquide surnageant. Pour faire les broyats on utilise 5 ou 20 queues.

Le Tableau 16 met en évidence que le pourcentage de planaires qui régénèrent les yeux est d'autant plus élevé que les broyats sont plus concentrés.

La comparaison entre les résultats expérimentaux des Tableaux 6 et 16 montre que le surnageant des broyats de têtes est plus actif que le surnageant des broyats de queues. Une tête de *P. nigra* doit contenir beaucoup plus d'organisine qu'une région caudale.

TABLEAU 16

Influence de la concentration des broyats de queues centrifugés

Concentration des broyats	*Planaires en expérience*	*Pas de régénération des yeux*	*Régénération des yeux*			*Régénération du cerveau*		*Régénération des yeux sous l'influence des broyats*
			Nbre de planaires	*Nbre d'yeux*	*Délai (en jours)*	*Nbre de planaires*	*Longueur (en μ)*	
5 queues .	12	10	2	2	9	0	—	2
20 queues .	12	5	7	1–3	7	1	100	6
Pas de broyats (témoins)	12	8	4	1–3	7	4	125 à 150	—

La différence d'activité, mise en évidence entre les deux types de broyats, est donc due non seulement à une immobilisation de l'organisine dans les cellules de la queue, mais aussi à une différence appréciable de concentration de la substance dans les deux parties du corps.

DISCUSSION DES RÉSULTATS ET CONCLUSION

(A) *L'Organisine de la régénération des yeux de* Polycelis *est une substance chimique*

De l'ensemble de ces expériences se dégage la conclusion que l'organisine de la régénération des yeux de *Polycelis nigra* est une substance chimique. L'organisateur conserve son pouvoir inducteur même après la lésion ou la mort du tissu. Celui-ci peut être tué par l'alcool ou un chauffage modéré; l'induction des yeux se fait normalement. Elle n'est donc pas liée à l'activité vitale des cellules.

L'organisine de la régénération des yeux est diffusible dans l'eau. Grâce à cette propriété on peut facilement l'extraire des broyats au sable.

La substance possède une certaine thermolabilité puisqu'un traitement à 100° pendant 30 minutes fait presque disparaître le pouvoir inducteur.

Elle ne possède pas de spécificité zoologique puisque des broyats de têtes d'autres planaires induisent aussi la régénération des yeux de *P. nigra*.

L'activité des solutions d'organisine dépend de leur concentration. Jusqu'à présent la détermination de cette concentration est encore empirique. Elle est exprimée en nombre de têtes de planaires broyées dans 10 c.c. d'eau. Pour obtenir, en présence de broyats, une régénération des yeux comparable à la régénération sous l'influence du cerveau in situ, il faut utiliser une solution contenant le broyat de 10 à 15 têtes dans 10 c.c. Si la concentration est plus faible le nombre d'individus régénérant les yeux est plus faible. Si la concentration est plus forte la régénération des yeux est accélérée de 24 à 48 heures. L'organisine

des solutions favorise peut-être la mise en place des cellules de régénération, mais elle induit sûrement la différenciation des néoblastes en cellules visuelles. Car la substance agit le plus efficacement vers les 3ème et 4ème jours après l'excision des ocelles, quand les néoblastes sont déjà en place dans le blastème de régénération.

(B) *Répartition de l'organisine dans le corps de la planaire*

Pendant la régénération normale des yeux, l'organisine est produite par le cerveau. En effet l'excision du cerveau de 420 témoins a montré que la régénération des yeux ne se produit pas si on empêche la différenciation de la masse cérébrale.

L'organisine diffuse librement dans la région antérieure du corps, c'est-à-dire dans la région normalement oculée. Le reste du corps de la planaire contient aussi une substance organisatrice. Mais celle-ci ne se manifeste pas tant que les cellules ont gardé leur intégrité. La substance est bloquée dans les cellules. Si on hache les régions postérieures, l'activité inductrice ne se manifeste pas. Mais si on tue les cellules, par exemple en chauffant les broyats à 80°, en broyant les cellules avec du sable, ou en faisant agir l'alcool à 70°, la substance inductrice est libérée.

La substance inductrice des yeux mise en évidence dans les broyats de queues semble être la même que celle qui est émise par le cerveau. Car les deux substances se comportent de la même manière dans les différentes expériences: action identique de la chaleur, de l'alcool, diffusion dans l'eau. Nous pouvons donc admettre qu'il n'y a qu'une organisine présente dans tout le corps de la planaire.

La différence d'efficacité des broyats de têtes et des broyats de queues n'est pas seulement due à la différence de mobilité de l'organisine mais aussi à une différence de concentration. La concentration de l'organisine dans les broyats de queues est plus faible que dans les broyats de têtes. Avec un broyat au sable de 20 queues, le nombre d'individus qui régénèrent les yeux est voisin du nombre qu'on obtient avec un broyat au sable de 5 têtes.

Il existe donc un gradient de concentration de l'organisine de la régénération des yeux, gradient décroissant de la tête vers la queue (Lender, 1954), qui se superpose au gradient métabolique de Child.

(C) *Comparaison de l'organisine et de l'inducteur du développement des batraciens*

Nous savons maintenant que la régénération des yeux de *Polycelis nigra* est induite par une substance chimique, une organisine. Or certains auteurs pensent que des inductions par voie chimique interviendraient dans le développement

embryonnaire des batraciens. La comparaison des deux processus met en évidence de nombreuses analogies.

Dans les deux cas l'inducteur n'a pas de spécificité zoologique; il est insensible à l'alcool, à la chaleur (Bautzmann, 1932; Holtfreter, 1932, 1933, 1934; Spemann, 1932). Mais l'inducteur du développement des batraciens semble être moins sensible à la chaleur que l'organisine de la régénération des yeux. Cependant, dans les deux cas, le pouvoir inducteur diminue si on chauffe l'organisateur.

Récemment Niu & Twitty (1953) ont montré qu'un liquide dans lequel avaient séjourné des explants mésodermiques de jeunes gastrula, provoquait une différenciation histologique de fragments d'ectoderme. Dans ce cas, comme dans la régénération des yeux de *Polycelis nigra*, l'induction se produit sans qu'il y ait contact physique entre le réacteur et l'inducteur. L'organisine a diffusé dans l'eau.

Enfin un dernier point de comparaison est l'éveil de l'activité inductrice dans un tissu normalement non inducteur. Si on tue par dessiccation ou chauffage un tissu comme l'entoderme ou l'épiderme présomptif de la gastrula de triton (Holtfreter, 1932, 1933, 1934) ces tissus deviennent inducteurs. Mais un non inducteur ainsi activé ne donne pas un rendement aussi élevé que l'inducteur normal. Or les expériences sur la régénération des yeux mettent en évidence les mêmes faits. La région caudale tuée par l'alcool, la chaleur ou le broyage au sable, devient inductrice. Mais ce pouvoir inducteur est, dans la majorité des cas, plus faible que celui de l'organisine des broyats de têtes.

Pour expliquer l'apparition du pouvoir inducteur dans les tissus activés de larves de batraciens, Holtfreter a pensé que les tissus auraient perdu leur perméabilité sélective, ce qui permettrait la diffusion de la substance inductrice. Toivonen (1940) a repris cette hypothèse en l'appliquant aux inducteurs hétérogènes activés. Or dans la régénération des yeux de *P. nigra* cette hypothèse semble bien se confirmer. Si on tue, par la chaleur ou l'alcool, les cellules d'un non inducteur, comme la queue, le tissu est activé et induit la régénération des yeux. Mais on obtient le même résultat si on fait simplement un broyage soigné au sable. Dans ce cas les cellules sont déchirées par les grains de sable et l'organisine peut sortir de la cellule. Il semble donc que le pouvoir inducteur apparaît dans certains tissus si on détruit la perméabilité sélective des cellules.

RÉSUMÉ

1. Dans ce travail j'ai étudié la régénération des yeux de la planaire *Polycelis nigra* (Ehrbg.) en l'absence du cerveau. L'apparition des ocelles a été observée si on élève les planaires en présence de broyats de têtes hachées, de broyats de têtes enrobées dans la gélose ou en présence du liquide surnageant obtenu par centrifugation de têtes broyées au sable. L'induction se fait donc grâce à une substance chimique, une organisine.

2. La régénération des yeux en l'absence du cerveau est possible si l'organisine est présente pendant un jour sur deux ou si elle n'agit que pendant deux jours.

Les meilleurs résultats sont obtenus si on fait agir la substance inductrice entre le 3ème et le 4ème jour après l'excision des ocelles.

3. La concentration de l'organisine joue un rôle dans la différenciation des ocelles. L'apparition des yeux est favorisée par l'augmentation de la concentration de l'organisine dans l'eau d'élevage.

4. Le traitement par l'alcool ne rend pas l'organisine inactive. Le traitement par la chaleur à 60° ne fait pas disparaître le pouvoir inducteur des broyats. Ceux-ci deviennent moins actifs si on élève la température. Une ébullition pendant 30 minutes rend l'organisine presque inactive.

5. L'organisine ne présente pas de spécificité zoologique. On peut la mettre en évidence dans la région cérébrale de *Dugesia lugubris*, *D. gonocephala* et *Dendrocoelum lacteum*.

6. Les broyats de régions pharyngiennes ou caudales hachées ne provoquent pas la régénération des yeux. Mais les broyats de queues peuvent être activés par traitement à 60° pendant 2 minutes. Si on chauffe plus fortement, le pouvoir inducteur diminue comme pour les broyats de têtes.

7. Les broyats de queues tuées par l'alcool sont aussi activés. De même le liquide surnageant de queues broyées au sable contient l'organisine.

8. Les broyats de queues sont d'autant plus actifs qu'ils ont été préparés à partir d'un plus grand nombre de régions caudales. Mais les broyats de queues contiennent moins d'organisine que les broyats de têtes.

9. La régénération des yeux de *Polycelis nigra* est donc induite par une substance chimique, une organisine, présente dans tout le corps de la planaire. Dans la région antérieure sa concentration est plus forte que dans la région postérieure où elle est en plus immobilisée dans les cellules. Ces résultats sont à rapprocher de ceux qui sont obtenus dans l'étude du développement embryonnaire des batraciens.

SUMMARY

1. After excision of eyes and brain of the planarian *Polycelis nigra* (Ehrbg.), regeneration of eyes fails unless the brain is allowed to regenerate too. But regeneration of eyes in the absence of the brain occurs if the animals are kept in the presence of a mince of planarian heads, or of such a mince embedded in agar, or of the supernatant obtained after grinding heads with sand. The induction of eye regeneration is therefore performed by a chemical substance, an organisine.

2. Regeneration of eyes in the absence of the brain is possible if the organisine is only allowed to act on alternate days, or only for two consecutive days in all. In the latter case, best results are obtained if the substance acts during the 3rd and 4th days after operation.

3. Regeneration of eyes is favoured by increased concentration of organisine in the medium.

4. Treating the inducing material with 70 per cent. alcohol does not inactivate the organisine, nor does warming to 60° for 2 minutes. Higher temperatures reduce its activity, and boiling for 30 minutes almost destroys it.

5. The organisine is not species-specific. Its presence can be demonstrated in the brain region of *Dugesia lugubris*, *D. gonocephala*, and *Dendrocoelum lacteum*.

6. A mince of the pharyngeal or caudal region does not produce regeneration of eyes. But a mince of the caudal region can be activated by treatment at 60° for 2 minutes. With stronger heating the inducing power diminishes, as with the mince of heads.

7. A mince of caudal regions is also activated if it is treated with alcohol of 70 or 95 per cent. The supernatant obtained from grinding caudal regions with sand also contains the organisine.

8. Such supernatants from the caudal regions are more active the greater the number of animals from which they are prepared; but they contain less organisine than the supernatant from an equivalent number of head regions.

9. The regeneration of eyes of *Polycelis nigra* is therefore induced by a chemical substance, an organisine, present throughout the body of the planarian. Its concentration is stronger in the anterior than in the posterior region; in the latter it is furthermore immobilized within the cells. These results are comparable with those obtained in the study of amphibian embryonic induction.

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(Manuscript received 7:x:55)

A Remarkable Case of Animalization in a Batch of Eggs of *Paracentrotus lividus*

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Dedicated to
PROFESSOR ALFRED KÜHN
on his 70th birthday, 22nd April 1955

SEA-URCHIN eggs generally develop quite normally after artificial fertilization. Sometimes it may be difficult to obtain good membrane elevation, but, after repeated washing in sea-water, fertilization and further development may be quite normal. In some batches of eggs abnormalities may, however, be observed in the pluteus stage, but these abnormalities, as a rule, do not change the pattern of the larvae profoundly. The defects are generally restricted to the arms and to the skeletal rods which, for example, may fail to grow out to their normal length, or may bend in an abnormal direction. Supernumerary rods may also appear, and rods may grow in an abnormal direction inside the larval body without causing a change in the exterior of the pluteus. During many years of experimental work with sea-urchin eggs, for the senior author dating thirty years back, we have never encountered, nor have we seen in the literature, examples of such an abnormal development as the one described in this paper.

In a batch of eggs of *Paracentrotus lividus* obtained in August 1954 at Roscoff we found that about 1 per cent. of the larvae did not gastrulate at all (Text-fig., I–III), or only formed a very small archenteron (Text-fig., IV–VII). In all cases a ciliary band was well developed, and as a rule a stomodaeum also, although the latter was missing in a few cases (Text-fig., I). In more than 50 per cent. of these abnormal larvae a very small archenteron had invaginated, forming a single or bipartite vesicle (Text-fig., IV–VII).

In order to interpret this abnormal development we refer to the fact that exactly these types of larvae have been described before, but never from whole eggs. They resemble larvae obtained by one of us (Hörstadius, 1935) from more or less animal egg fragments of *Paracentrotus*. Isolated animal halves do not gastrulate, but develop into one of the following types: blastulae uniformly covered with cilia (type A), blastulae with a ciliated field and the rest of the wall consisting of a pavement epithelium (type B), blastulae with a ciliated band (type

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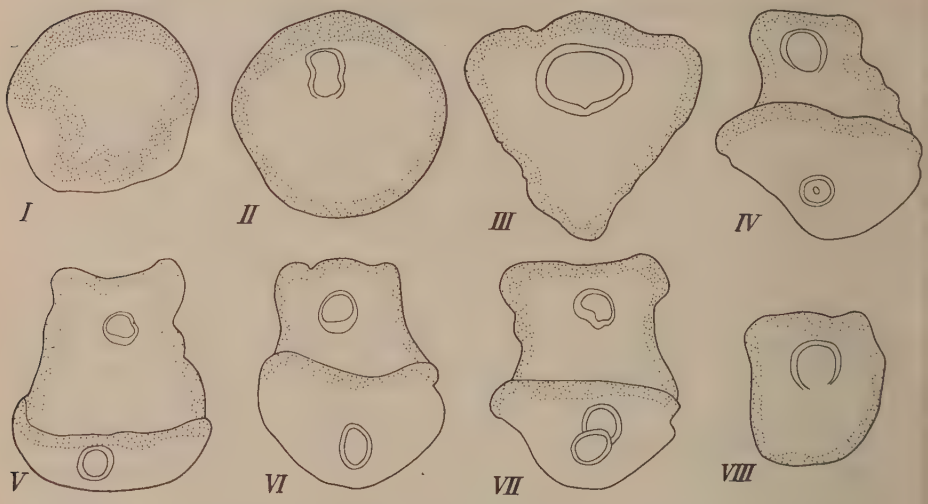
[J. Embryol. exp. Morph. Vol. 4, Part 2, pp. 217–19, June 1956]

C), or blastulae with both ciliated band and stomodaeum (type D). The explanation given for the genesis of these types runs as follows. The absence of the



TEXT-FIG. Camera lucida drawings of abnormal larvae (I–VII) of *Paracentrotus lividus*. VIII, an isolated animal half of the same type as larvae II and III, drawn at the same magnification.

vegetal half causes a predominance of the animal properties, leading to an increase of the apical tuft and lack of differentiation of a thin epithelium and a stomodaeum. The halves of type A are to be regarded as the most animalized. In the other types the vegetal properties have been more strongly represented and favoured, e.g. the development of both a ciliated band and a stomodaeum (type D), a differentiation equal to the prospective significance of the material.

Animal halves giving the richer differentiation have been called 'subequatorial', because in some batches the third furrow, in the eighth-cell stage, is situated below the equator. In such eggs the animal halves will contain more vegetal material and consequently have stronger vegetal properties than halves from 'equatorial' eggs. This explains the more normal differentiation. It must be emphasized, however, that such a 'vegetal' development can also be obtained in eggs which are strictly 'equatorial' in their cleavage. This indicates that the balance between the animal and the vegetal gradients is not the same in all eggs. A further proof of this is afforded by a study of fragments called $8 + veg_1 + 0$ (op. cit.), i.e. fragments containing not only the cells from the animal half (8 mesomeres in the 16-cell stage), but also the adjacent ring of eight blastomeres (veg_1) from the vegetal half in the 64-cell stage. This material, the animal half + veg_1, corresponds to the entire ectoderm of the pluteus larva. In this experiment we have added some vegetal material to the animal half and the result is as

would be expected, that ciliated band and stomodaeum also appear in fragments derived from 'equatorial' batches of eggs. 'Subequatorial' fragments show a more vegetal differentiation even if the third cleavage furrow is equatorial, as a small archenteron and even skeletal spicules appear (op. cit., fig. 25).

It is now of particular interest that the abnormal larvae obtained from whole eggs (Text-fig., I–VII) have their exact counterparts in animal fragments described in the paper of 1935. Only the size is different. Our blastula with ciliated band (I) is of the same type as the isolated animal half (fig. 12*c*, op. cit.). II and III correspond to the animal halves in fig. 13 *a–c*, or the 'equatorial' $8 + \text{veg}_1 + 0$-fragments in fig. 24 *f–h*. A 'subequatorial' fragment like that in fig. 25*a* is similar to our larvae IV–VII.

The differentiation of these strange types cannot be due to a dropping off of the vegetal quarter of the egg in an early cleavage stage. The eggs developed inside their fertilization membrane, and no small larvae of any kind were found. Furthermore, the size indicates that the larva originates from a whole egg: compare the animal half (Text-fig., VIII) of the same type as II or III and drawn with the camera lucida at the same magnification.

The explanation must be that in these eggs the vegetal properties have been exceedingly weak compared with the animal ones. The gradient system in the whole egg has been of about the same constitution as normally found in fragments representing only the presumptive ectoderm ($8 + \text{veg}_1 + 0$). The cell physiological background for this situation is unknown. It may be mentioned in this connexion that about 1 per cent. animalization has been obtained in eggs of *Paracentrotus* treated for 10 hours before fertilization with calcium-free sea-water. Eggs from different localities reacted differently (Lindahl, Swedmark, & Lundin, 1951). It is quite possible that in this case the animalization was the result of the long treatment of the eggs with a calcium-free medium. It is interesting to find that the same effects occur spontaneously in eggs fertilized without any delay and kept in normal sea-water.

SUMMARY

In 1954 at Roscoff some larvae from a batch of eggs of *Paracentrotus lividus* were found to develop in the same way as eggs from which the entire endo- and mesodermal region had been removed. The effect occurred quite spontaneously.

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(Manuscript received 9:xi:55)

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Journal of
Embryology and Experimental Morphology

[J. Embryol. exp. Morph.]

VOLUME 4 *June 1956* PART 2

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PRINTED IN GREAT BRITAIN, AT THE UNIVERSITY PRESS, OXFORD
BY CHARLES BATEY, PRINTER TO THE UNIVERSITY